Application
for
United States Letters Patent

To all whom it may concern:

Be it known that we,

Christophe P.G. Gerald, Kenneth A. Jones, James A. Bonini and Beth Borowsky

have invented certain new and useful improvements in

DNA ENCODING MAMMALIAN NEUROPEPTIDE FF (NPFF) RECEPTORS AND USES THEREOF

of which the following is a full, clear and exact description.

Dkt. 57155-A/JPW/JHB

DNA ENCODING MAMMALIAN NEUROPEPTIDE FF (NPFF) RECEPTORS AND USES THEREOF

BACKGROUND OF THE INVENTION

INSAI

[01] ~~This application is a continuation-in-part of U.S. Serial No. 09/161,113, filed September 25, 1998 the contents of which are hereby incorporated by reference into the subject application.~~

Throughout this application, various publications are referenced in parentheses by author and year. Full citations for these references may be found at the end of the specification immediately preceding the sequence listings and the claims. The disclosure of these publications in their entireties are hereby incorporated by reference into this application to describe more fully the art to which this invention pertains.

Neuroregulators comprise a diverse group of natural products that subserve or modulate communication in the nervous system. They include, but are not limited to, neuropeptides, amino acids, biogenic amines, lipids and lipid metabolites, and other metabolic byproducts. Many of these neuroregulator substances interact with specific cell surface receptors which transduce signals from the outside to the inside of the cell. G-protein coupled receptors (GPCRs) represent a major class of cell surface receptors with which many neurotransmitters interact to mediate their effects. GPCRs are predicted to have seven membrane-spanning domains and are coupled to their effectors via G-proteins linking receptor activation with intracellular biochemical sequelae such as stimulation of adenylyl cyclase.

Neuropeptide FF (NPFF) is an octapeptide isolated from bovine brain in 1985 by Yang and coworkers (1) using antibodies to the molluscan neuropeptide FMRFamide (FMRFa). FMRFamide-like immunoreactivity was observed in rat brain, spinal cord, and pituitary, suggesting the existence of mammalian homologs of the FMRFa family of invertebrate peptides. The isolation of NPFF, named for its N- and C-terminal phenylalanines (also called F8Famide) and a second mammalian peptide, NPAF (also called A18Famide), confirmed the existence of mammalian family of peptides sharing C-terminal sequence homology with FMRFa (1). Molecular cloning has revealed that NPFF and NPAF are encoded by the same gene and cleaved from a common precursor protein (2). Studies of the localization, radioligand binding, and function of NPFF-like peptides (see below) indicate they are neuromodulatory peptides whose effects are likely to be mediated by G protein-coupled receptors (for review, see 3).

NPFF, also called "morphine modulating peptide", is an endogenous modulator of opioid systems with effects on morphine analgesia, tolerance, and withdrawal (for review see 3,4). NPFF appears to represent an endogenous "anti-opioid" system in the CNS acting at specific, high-affinity receptors distinct from opiate receptors (5,6). Endogenous NPFF has been suggested to play a role in morphine tolerance: agonists of NPFF precipitate "morphine abstinence syndrome" (i.e. symptoms of morphine withdrawal) in morphine-dependent animals (7,8), while antagonists and anti-NPFF IgG restore morphine sensitivity and ameliorate symptoms of withdrawal (9-12). NPFF antagonists potentially could be useful as therapeutic agents to prevent the development of morphine tolerance,

and to treat opiate addiction. NPFF has also been suggested to participate in the regulation of pain threshold, showing both "anti-opiate" effects and analgesic effects depending on test system and route of administration (for review, see 4). As an anti-opiate, NPFF has been shown to inhibit morphine- and stress-induced analgesia (1, 13, 14, 15), whereas anti-NPFF IgG (which blocks the biological activity of NPFF) potentiates these two phenomena (16, 17). An NPFF antagonist may be clinically useful in potentiating the analgesic effects of morphine, allowing use of lower doses without the development of tolerance. NPFF agonists may also exhibit analgesic activity in some model systems (14, 18, 19). The analgesia elicited by NPFF is typically sensitive to naloxone, indicating that it is mediated by release of endogenous opioid peptides (19, 20). The interaction of NPFF and opioid systems in regulating pain pathways is thus complex and may involve multiple mechanisms and sites of action. NPFF has additional biological activities in accord with its pattern of expression in the nervous system.

NPFF peptide localization in rat CNS was examined using specific antibodies ((21-23); see also (3)). The highest levels of NPFF are found in spinal cord and posterior pituitary; pituitary NPFF is believed to originate in the hypothalamus. In the brain, immunoreactive cell bodies are found in two major cell groups: medial hypothalamus (between dorsomedial and ventromedial) and nucleus of the solitary tract. Immunoreactive fibers are observed in lateral septal nucleus, amygdala, hypothalamus, nucleus of solitary tract, ventral medulla, trigeminal complex, and dorsal horn of spinal cord. This localization pattern is consistent with a role for NPFF in sensory processing and

modulation of opioid systems. In addition, its presence in the hypothalamus and other limbic structures could subserve roles in the regulation of appetitive and affective states. In the periphery, NPFF-like immunoreactivity (as well as NPFF binding) has been observed in the heart (24). In addition, injection of NPFF raises blood pressure in rats (24, 25). These observations, combined with the colocalization of NPFF with catecholaminergic neurons in the nucleus of the solitary tract (26), suggest that NPFF is involved in central and peripheral cardiovascular regulation.

The ability of NPFF peptides to modulate the opioid system raised the possibility that NPFF interacts directly with opiate receptors. However, radioligand binding assays using a tyrosine-substituted NPFF analog [^{125}I]Y8Fa demonstrate that NPFF acts through specific high affinity binding sites distinct from opiate receptors (27-30) that are sensitive to inhibition by guanine nucleotides (31). The latter observation indicates that NPFF receptors are likely to belong to the superfamily of G protein-coupled receptors which share common structural motifs. However, no reports of cloning NPFF receptors have appeared as yet.

To address the issue of potential degradation of the peptide radioligand, a more stable NPFF analog (called (1DMe)Y8Fa(18)) has also been radioiodinated and the binding characterized in spinal cord membranes (32). The binding was saturable and of high affinity; inhibition of binding with unlabeled NPFF analogs yielded Ki values of 0.16nM and 0.29nM for (1DMe)Y8Fa and NPFF, respectively, with a Bmax = 15 fmol/mg protein. No inhibition by various opioid compounds (naloxone, morphine, enkephalins, dynorphins, etc.) or other peptides (NPY, SP, CGRP, for

examples) was observed at a concentration of 10 μM, confirming the specificity of NPFF receptors. Interestingly, the related molluscan peptide FMRFa inhibited the binding of [^{125}I](1DMe)Y8Fa with a Ki = 30 nM. The effectiveness of FMRFamide and the C-terminal fragment NPFF(6-8) at NPFF receptors suggests an important role for the common C-terminus. Full activity is retained by NPFF (3-8); it has been suggested that although the C-terminus is important for receptor recognition, the N-terminus is necessary for formation of a high-affinity conformation (33).

Allard et al. (29) examined the distribution of NPFF binding sites in rat brain and spinal cord using [^{125}I]Y8Fa ([^{125}I]YLFQPQRFamide). The highest densities were observed in the external layers of dorsal horn of spinal cord, several brainstem nuclei, the suprachiasmatic nucleus, restricted nuclei of the thalamus, and the presubiculum of the hippocampus. Lower densities were seen in central gray, reticular formation, ventral tegmental area, lateral and anterior hypothalamus, medial preoptic area, lateral septum, the head of caudate-putamen and cingulate cortex. No binding was observed in cortex, nucleus accumbens, hippocampus (except in presubiculum) or cerebellum. The localization of NPFF binding sites is in good agreement with the location of the peptide itself, consistent with the binding sites mediating the biological actions of NPFF in these tissues (29, 34, 35). Less is known about the signal transduction pathways activated by NPFF receptors; NPFF was shown to activate adenylyl cyclase in mouse olfactory bulb membranes (36) but no other reports of functional coupling via G proteins have appeared.

Until now, no direct evidence for NPFF receptor subtypes has been reported in mammals. Recent physiological data suggest complex (biphasic) effects on nociception and antiopiate activity of NPFF (for review, see (3, 4)) that could possibly signal the presence of multiple subtypes. Short term ICV injection of NPFF causes a hyperesthesic effect followed by long lasting analgesic effect. Intrathecal NPFF and FMRFa both produce long-lasting analgesia, but subeffective doses caused different modulatory effects on morphine-induced analgesia (F8Fa potentiated, FMRFa decreased). The analgesic effects of NPFF are sensitive to naloxone, suggesting that NPFF receptors may have distinct presynaptic (increase release of opioids?) and postsynaptic (anti-opiate?) effects mediated by multiple receptors. Little is known of the biological effects of A18Famide, which shares its C-terminal 4 amino acids with NPFF, but the existence of a family of related peptides often is predictive of multiple receptor subtypes.

No nonpeptide agonists or antagonists of NPFF are available, but several useful peptidic analogs have been developed that exhibit increased agonist stability or antagonist activity. For example, desamino Y8Fa (daY8Fa) can antagonize the behavioral effects of NPFF and restore morphine-sensitivity (tail-flick analgesia) to morphine-tolerant rats at lower doses, although at higher doses it can act as NPFF agonist (10)(see also (3)). (1DMe)Y8Fa, in which L-Phe^1 is replaced by D-Tyr and the second peptidic bond is N-methylated, has been shown to inhibit morphine-induced analgesia (18), and has higher affinity and stability than Y8Fa: (1DMe)Y8Fa was 90% stable after 150 min. incubation with rat spinal cord membranes compared with Y8Fa, which was fully degraded after 30

minutes. These analogs may be useful in predicting the effects of agonist or antagonist drugs that would act at NPFF receptors.

Despite the numerous studies linking NPFF with analgesia (for review, see (4)), only recently has NPFF been observed to play a role in animal models of chronic pain. For example, NPFF has recently been shown to be involved in inflammatory pain (37) and neuropathic pain (38). Importantly, NPFF was shown to attenuate the allodynia associated with neuropathic pain, suggesting that it may be clinically useful in treating this condition. In addition to its potential therapeutic roles in the treatment of pain and morphine tolerance ((4) and above), NPFF and related peptides have a number of other biological activities that may be therapeutically relevant including effects on feeding (39-41), psychotic behavior (42), nicotine addiction (43), and cardiovascular functions (24, 25). The cloning of NPFF receptors will facilitate the elucidation of the roles of NPFF and related peptides in these and other important biological functions.

Described herein is the isolation and characterization of a new family of neuropeptide FF (NPFF) receptors, referred to herein as the NPFF receptors. Cloned NPFF receptors will serve as invaluable tools for drug design for pathophysiological conditions such as memory loss, affective disorders, schizophrenia, pain, hypertension, locomotor problems, circadian rhythm disorders, eating/body weight disorders, sexual/reproductive disorders, nasal congestion, diarrhea, gastrointestinal, and cardiovascular disorders.

SUMMARY OF THE INVENTION

This invention provides an isolated nucleic acid encoding a mammalian NPFF receptor.

This invention provides a nucleic acid encoding a mammalian NPFF receptor, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figure 1 (Seq. ID No. 1) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its complement. This invention further provides a nucleic acid encoding a mammalian NPFF receptor, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figure 4 (Seq. ID No. 3) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its complement. This invention also provides a nucleic acid encoding a mammalian NPFF receptor, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figure 7 (Seq. ID No. 5) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its

complement.

This invention further provides a nucleic acid encoding a mammalian NPFF receptor, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figure 11 (Seq. ID No. 7) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its complement.

This invention also provides a purified mammalian NPFF receptor protein.

This invention further provides a vector comprising a nucleic acid encoding a mammalian NPFF receptor, particularly a vector adapted for expression of the mammalian NPFF receptor in mammalian or non-mammalian cells.

This invention provides a plasmid designated pEXJ-rNPFF1 (ATCC Accession No. 203184). This invention also provides a plasmid designated pWE15-hNPFF1 (ATCC Accession No. 203183). This invention further provides a plasmid designated pCDNA3.1-hNPFF2b (ATCC Accession No. 203255). This invention still further provides a plasmid designated pcDNA3.1-hNPFF1 (ATCC Accession No. 203605).

This invention additionally provides a cell comprising a vector which in turn comprises a nucleic acid encoding a mammalian NPFF receptor as well as a membrane preparation isolated from such a cell.

Moreover, this invention provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within one of the two strands of the nucleic acid encoding the mammalian NPFF1 receptor and contained in plasmid pEXJ-rNPFF1 (ATCC Accession No. 203184), plasmid pWE15-hNPFF1 (ATCC Accession No. 203183), plasmid pCDNA3.1-hNPFF2b (ATCC Accession No. 203255), or plasmid pcDNA3.1-hNPFF1 (ATCC Accession No. 203605).

This invention further provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 1(Seq. I.D. No. 1) or (b) the reverse complement thereto.

This invention further provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 4 (Seq. I.D. No. 3) or (b) the reverse complement thereto.

This invention further provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 7 (Seq. I.D. No.

5) or (b) the reverse complement thereto.

This invention further provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 11 (Seq. I.D. No. 7) or (b) the reverse complement thereto.

This invention still further provides an antisense oligonucleotide having a sequence capable of specifically hybridizing to RNA encoding the mammalian NPFF receptor, so as to prevent translation of the RNA. This invention also provides an antisense oligonucleotide having a sequence capable of specifically hybridizing to genomic DNA encoding a mammalian NPFF receptor, so as to prevent transcription thereof.

This invention further provides an antibody capable of binding to a mammalian NPFF receptor. This invention also provides an agent capable of competitively inhibiting the binding of the antibody to a mammalian NPFF receptor.

In addition, this invention provides a pharmaceutical composition comprising (a) an amount of the oligonucleotide described above capable of passing through a cell membrane and effective to reduce expression of a mammalian NPFF receptor and (b) a pharmaceutically acceptable carrier capable of passing through the cell membrane.

This invention also provides a transgenic, nonhuman mammal expressing DNA encoding a mammalian NPFF receptor. This

invention also provides a transgenic, nonhuman mammal comprising a homologous recombination knockout of the native mammalian NPFF receptor. This invention further provides a transgenic, nonhuman mammal whose genome comprises antisense DNA complementary to the DNA encoding a mammalian NPFF receptor so placed within the genome as to be transcribed into antisense mRNA which is complementary to mRNA encoding the mammalian NPFF receptor and which hybridizes to mRNA encoding the mammalian NPFF receptor, thereby reducing its translation.

This invention provides a process for identifying a chemical compound which specifically binds to a mammalian NPFF receptor which comprises contacting cells containing DNA encoding and expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the mammalian NPFF receptor.

This invention further provides a process for identifying a chemical compound which specifically binds to a mammalian NPFF receptor which comprises contacting a membrane preparation from cells transfected with DNA encoding and expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the mammalian NPFF receptor.

This invention provides a process involving competitive binding for identifying a chemical compound which

specifically binds to a mammalian NPFF receptor which comprises separately contacting cells expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the mammalian NPFF receptor, a decrease in the binding of the second chemical compound to the mammalian NPFF receptor in the presence of the chemical compound indicating that the chemical compound binds to the mammalian NPFF receptor.

This invention further provides a process involving competitive binding for identifying a chemical compound which specifically binds to a mammalian NPFF receptor which comprises separately contacting a membrane fraction from cells expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the mammalian NPFF receptor, a decrease in the binding of the second chemical compound to the mammalian NPFF receptor in the presence of the chemical compound indicating that the chemical compound binds to the mammalian NPFF receptor.

This invention provides a method of screening a plurality of chemical compounds not known to bind to a mammalian NPFF receptor to identify a compound which specifically

binds to the mammalian NPFF receptor, which comprises (a) contacting cells transfected with and expressing DNA encoding the mammalian NPFF receptor with a compound known to bind specifically to the mammalian NPFF receptor; (b) contacting the preparation of step (a) with the plurality of compounds not known to bind specifically to the mammalian NPFF receptor, under conditions permitting binding of compounds known to bind to the mammalian NPFF receptor; (c) determining whether the binding of the compound known to bind to the mammalian NPFF receptor is reduced in the presence of any compound within the plurality of compounds, relative to the binding of the compound in the absence of the plurality of compounds; and if so (d) separately determining the binding to the mammalian NPFF receptor of compounds included in the plurality of compounds, so as to thereby identify the compound which specifically binds to the mammalian NPFF receptor.

This invention also provides a method of screening a plurality of chemical compounds not known to bind to a mammalian NPFF receptor to identify a compound which specifically binds to the mammalian NPFF receptor, which comprises (a) contacting a membrane preparation from cells transfected with and expressing DNA encoding a mammalian NPFF receptor with a compound known to bind to the mammalian NPFF receptor; (b) determining whether the binding of a compound known to bind to the mammalian NPFF receptor is reduced in the presence of any compound within the plurality of compounds, relative to the binding of the compound in the absence of the plurality of compounds; and if so (c) separately determining the binding to the mammalian NPFF receptor of compounds included in the plurality of compounds, so as to thereby identify the

compound which specifically binds to the mammalian NPFF receptor.

Still further, this invention provides a method of detecting expression of a mammalian NPFF receptor by detecting the presence of mRNA coding for the mammalian NPFF receptor which comprises obtaining total mRNA from the cell and contacting the mRNA so obtained with a nucleic acid probe under hybridizing conditions, detecting the presence of mRNA hybridizing to the probe, and thereby detecting the expression of the mammalian NPFF receptor by the cell.

This invention provides a method of detecting the presence of a mammalian NPFF receptor on the surface of a cell which comprises contacting the cell with an antibody under conditions permitting binding of the antibody to the receptor, detecting the presence of the antibody bound to the cell, and thereby detecting the presence of the mammalian NPFF receptor on the surface of the cell.

This invention provides a method of determining the physiological effects of varying levels of activity of mammalian NPFF receptors which comprises producing a transgenic, nonhuman mammal whose levels of mammalian NPFF receptor activity are varied by use of an inducible promoter which regulates mammalian NPFF receptor expression.

This invention also provides a method of determining the physiological effects of varying levels of activity of mammalian NPFF receptors which comprises producing a panel of transgenic, nonhuman mammals each expressing a different amount of mammalian NPFF receptor.

This invention further provides a method for identifying an antagonist capable of alleviating an abnormality wherein the abnormality is alleviated by decreasing the activity of a mammalian NPFF receptor comprising administering a compound to a transgenic, nonhuman mammal as described above and determining whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic, nonhuman mammal as a result of overactivity of a mammalian NPFF receptor, the alleviation of the abnormality identifying the compound as an antagonist. This invention also provides an antagonist identified by this method. This invention still further provides a pharmaceutical composition comprising an antagonist identified by this method and a pharmaceutically acceptable carrier.

This invention additionally provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by decreasing the activity of a mammalian NPFF receptor which comprises administering to the subject an effective amount of the preceding pharmaceutical composition containing a mammalian NPFF receptor antagonist, thereby treating the abnormality.

This invention also provides a method for identifying an agonist capable of alleviating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a mammalian NPFF receptor comprising administering a compound to a transgenic, nonhuman mammal, and determining whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic, nonhuman mammal, the alleviation of the abnormality identifying the compound as an agonist. This invention also provides an agonist identified by this

method. This invention further provides a pharmaceutical composition comprising an agonist identified by this method and a pharmaceutically acceptable carrier. This invention provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a mammalian NPFF receptor which comprises administering to the subject an effective amount of the preceding pharmaceutical composition containing a mammalian NPFF receptor agonist, thereby treating the abnormality.

This invention provides a method for diagnosing a predisposition to a disorder associated with the activity of a specific mammalian allele which comprises: (a) obtaining DNA of subjects suffering from the disorder; (b) performing a restriction digest of the DNA with a panel of restriction enzymes; (c) electrophoretically separating the resulting DNA fragments on a sizing gel; (d) contacting the resulting gel with a nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a mammalian NPFF receptor and labeled with a detectable marker; (e) detecting labeled bands which have hybridized to the DNA encoding a mammalian NPFF receptor labeled with a detectable marker to create a unique band pattern specific to the DNA of subjects suffering from the disorder; (f) preparing DNA obtained for diagnosis by steps (a)-(e); and (g) comparing the unique band pattern specific to the DNA of subjects suffering from the disorder from step (e) and the DNA obtained for diagnosis from step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to the disorder if the patterns are the same.

This invention provides a method of preparing a purified mammalian NPFF receptor which comprises: (a) culturing cells which express the mammalian NPFF receptor; (b) recovering the mammalian NPFF receptor from the cells; and (c) purifying the mammalian NPFF receptor so recovered.

This invention provides a method of preparing a purified mammalian NPFF receptor which comprises: (a) inserting a nucleic acid encoding the mammalian NPFF receptor into a suitable vector; (b) introducing the resulting vector into a suitable host cell; (c) placing the resulting cell in suitable condition permitting the production of the mammalian NPFF receptor; (d) recovering the mammalian NPFF receptor produced by the resulting cell; and (e) isolating and/or purifying the mammalian NPFF receptor so recovered.

This invention provides a process for determining whether a chemical compound is a mammalian NPFF receptor agonist which comprises contacting cells transfected with and expressing DNA encoding the mammalian NPFF receptor with the compound under conditions permitting the activation of the mammalian NPFF receptor, and detecting an increase in mammalian NPFF receptor activity, so as to thereby determine whether the compound is a mammalian NPFF receptor agonist. This invention also provides a pharmaceutical composition which comprises an amount of a mammalian NPFF receptor agonist determined by this process effective to increase activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier.

This invention provides a process for determining whether a chemical compound is a mammalian NPFF receptor antagonist which comprises contacting cells transfected with and expressing DNA encoding the mammalian NPFF

receptor with the compound in the presence of a known mammalian NPFF receptor agonist, under conditions permitting the activation of the mammalian NPFF receptor, and detecting a decrease in mammalian NPFF receptor activity, so as to thereby determine whether the compound is a mammalian NPFF receptor antagonist. This invention also provides a pharmaceutical composition which comprises an amount of a mammalian NPFF receptor antagonist determined by this process effective to reduce activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier.

This invention provides a process for determining whether a chemical compound specifically binds to and activates a mammalian NPFF receptor, which comprises contacting cells producing a second messenger response and expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with the chemical compound under conditions suitable for activation of the mammalian NPFF receptor, and measuring the second messenger response in the presence and in the absence of the chemical compound, a change in the second messenger response in the presence of the chemical compound indicating that the compound activates the mammalian NPFF receptor. This invention also provides a compound determined by this process. This invention further provides a pharmaceutical composition which comprises an amount of the compound (a NPFF receptor agonist) determined by this process effective to increase activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier.

This invention provides a process for determining whether a chemical compound specifically binds to and inhibits

activation of a mammalian NPFF receptor, which comprises separately contacting cells producing a second messenger response and expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with both the chemical compound and a second chemical compound known to activate the mammalian NPFF receptor, and with only the second chemical compound, under conditions suitable for activation of the mammalian NPFF receptor, and measuring the second messenger response in the presence of only the second chemical compound and in the presence of both the second chemical compound and the chemical compound, a smaller change in the second messenger response in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound indicating that the chemical compound inhibits activation of the mammalian NPFF receptor. This invention also provides a compound determined by this process. This invention further provides a pharmaceutical composition which comprises an amount of the compound (a mammalian NPFF receptor antagonist) determined by this effective to reduce activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier.

This invention provides a method of screening a plurality of chemical compounds not known to activate a mammalian NPFF receptor to identify a compound which activates the mammalian NPFF receptor which comprises: (a) contacting cells transfected with and expressing the mammalian NPFF receptor with the plurality of compounds not known to activate the mammalian NPFF receptor, under conditions permitting activation of the mammalian NPFF receptor; (b) determining whether the activity of the mammalian NPFF receptor is increased in the presence of the compounds;

and if so (c) separately determining whether the activation of the mammalian NPFF receptor is increased by each compound included in the plurality of compounds, so as to thereby identify the compound which activates the mammalian NPFF receptor. This invention also provides a compound identified by this method. This invention further provides a pharmaceutical composition which comprises an amount of the compound (a mammalian NPFF receptor agonist) identified by this method effective to increase activity of a mammalian NPFF receptor and a pharmceutically acceptable carrier.

This invention provides a method of screening a plurality of chemical compounds not known to inhibit the activation of a mammalian NPFF receptor to identify a compound which inhibits the activation of the mammalian NPFF receptor, which comprises: (a) contacting cells transfected with and expressing the mammalian NPFF receptor with the plurality of compounds in the presence of a known mammalian NPFF receptor agonist, under conditions permitting activation of the mammalian NPFF receptor; (b) determining whether the activation of the mammalian NPFF receptor is reduced in the presence of the plurality of compounds, relative to the activation of the mammalian NPFF receptor in the absence of the plurality of compounds; and if so (c) separately determining the inhibition of activation of the mammalian NPFF receptor for each compound included in the plurality of compounds, so as to thereby identify the compound which inhibits the activation of the mammalian NPFF receptor. This invention also provides a compound identified by this method. This invention further provides a pharmaceutical composition which comprises an amount of the compound (a mammalian NPFF receptor antagonist) identified by this process effective to

decrease activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier.

This invention provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a mammalian NPFF receptor which comprises administering to the subject an amount of a compound which is a mammalian NPFF receptor agonist effective to treat the abnormality.

This invention provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by decreasing the activity of a mammalian NPFF receptor which comprises administering to the subject an amount of a compound which is a mammalian NPFF receptor antagonist effective to treat the abnormality.

This invention provides a process for making a composition of matter which specifically binds to a mammalian NPFF receptor which comprises identifying a chemical compound using any of the processes described herein for identifying a compound which binds to and/or activates or inhibits activation of a mammalian NPFF receptor and then synthesizing the chemical compound or a novel structural and functional analog or homolog thereof. This invention further provides a process for preparing a pharmaceutical composition which comprises admixing a pharmaceutically acceptable carrier and a pharmaceutically acceptable amount of a chemical compound identified by any of the processes described herein for identifying a compound which binds to and/or activates or inhibits activation of a mammalian NPFF receptor or a novel structural and functional analog or homolog thereof.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1

Nucleotide sequence encoding a rat neuropeptide FF receptor (NPFF1) (Seq. I.D. No. 1). In addition, partial 5′ and 3′ untranslated sequences are shown. In Figure 1, two start (ATG) codons (at positions 73-75 and 148-150) and the stop (TAG) codon (at positions 1369-1371) are underlined.

Figure 2

Deduced amino acid sequence (Seq. I.D. No. 2) of the rat neuropeptide FF receptor (NPFF1) encoded by the nucleotide sequence shown Figures 1 (Seq. I.D. No. 1).

Figure 3

Deduced amino acid sequence for rat NPFF1 (SEQ. I.D. No. 2). Seven solid lines designated I-VII located above portions of the sequence indicate the seven putative transmembrane (TM) spanning regions.

Figure 4

Partial coding sequence of human neuropeptide FF receptor (NPFF1) (SEQ. I.D. No. 3).

Figure 5

Partial deduced amino acid sequence of the human neuropeptide FF (NPFF1) receptor (SEQ. I.D. No. 4) encoded by the partial nucleotide sequence of Figure 3.

Figure 6

Partial amino acid alignment of rat and human NPFF1. Vertical lines represent identical residues and dots represent similar residues.

Figure 7
Nucleotide sequence of hNPFF2b (SEQ. I.D. No. 5). The initiating methionine and the stop codon are underlined.

Figure 8
Deduced amino acid sequence of human NPFF2b (hNPFF2) (Seq. I.D. No. 6) encoded by the nucleotide sequence shown in Figure 7.

Figure 9
Deduced amino acid sequence for human hNPFF2 (SEQ. I.D. No. 6), with potential transmembrane domains underlined.

Figure 10
Amino acid alignment of rat NPFF1 and human NPFF2. Vertical lines represent identical residues and dots represent similar residues.
Figure Legends

Figure 11
Nucleotide sequence of a human neuropeptide FF receptor (NPFF1)(Seq. I.D. No. 7). The initiating methionine (at positions 1-3) and the stop codon (at positions 1291-1293) are underlined.

Figure 12
Deduced amino acid sequence of the human neuropeptide FF receptor (NPFF1)(Seq. I.D. No. 8).

Figure 13
Deduced amino acid sequence for human NPFF1 (Seq. I.D. No. 8). Seven solid lines designated I-VII indicate the seven putative transmembrane (TM) spanning regions.

Figure 14

Amino acid alignment of the human NPFF1 and human NPFF2 receptors. Vertical lines represent identical residues and dots represent similar residues.

Figure 15A-15C

Electrophysiological responses to NPFF and related peptides from voltage clamped oocytes expressing NPFF1 and chimeric G-protein.

Figures 16A-16C

Electrophysiological responses in voltage-clamped oocytes expressing NPFF2 mRNA. **Figure 16A:** Oocyte injected with NPFF2 mRNA (from ligation PCR) generates an inward current in response to NPFF at 1 μM. **Figure 16B:** In a different oocyte, no response is observed when challenged with a mixture of galanin, NPY, orexin A and neurokinin A, each at 1 μM. A subsequent application of NPFF elicits a response. **Figure 16C:** Oocyte injected with NPFF2 mRNA (from BO89) generates an inward current in response to NPFF at 1 μM. Oocytes were clamped at a holding potential of -80mV.

Figures 17A and 17B

Microphysiometric response of CHO cells transiently transfected with either NPFF1 (SN2) alone or NPFF1 accompanied by Gq/Gz. **Figure 17A:** Cells expressing either NPFF1 alone or NPFF1+Gq/Gz produced a dose-dependent response to NPFF with an EC50 value of 19.3 nM and 27.7 nM respectively. Mock control cells transfected with empty vector produced little if any response to NPFF even at the highest concentrations used. **Figure 17B:** Cells expressing NPFF1 alone produced a dose-dependent response to A-18-F-amide with an EC50 value of 150nM. In

both Figures 17A and 17B control cells mock transfected with empty vector produced little if any response to drug even at the highest concentrations used. Responses are reported as percentage increase in the acidification rate as observed just prior to drug challenge (immediate prior basal rate).

Figures 18A and 18B

NPFF stimulation of Inositol phosphate release in NPFF-1 transfected Cos-7 cells. **Figure 18A:** Cos-7 cells were transiently transfected with NPFF-1 receptor cDNA. **Figure 18B:** Cos-7 cells were transiently co-transfected with cDNAs for the NPFF-1 receptor and the Gq/Gz chimera. The accumulation of total inositol phosphate release was measured by prelabelling cells with [^{3}H]myoinositol (2μCi/ml) overnight. Cells were washed to remove unincorporated radioactivity and resuspended in medium containing 10mM LiCl. [^{3}H]myoinositol labeled cells were incubated with appropriate drugs for 1hr at 37°C. The reaction was stopped by addition of 5% TCA and IPs were isolated by ion exchange chromatography (Berridge et al., 1982). Columns were washed with water and total [^{3}H] inositol phosphates were then eluted with 1M ammonium formate/0.1 M formic acid. Radioactivity in the final fraction was measured by liquid scintillation spectroscopy. Cells were either treated with vehicle (water, control) or cholera toxin (CTX; 1 μg/ml) or pertussis toxin (PTX, 100 ng/ml) overnight. Data are from one experiment representative of at least one other.

Figure 19

RT-PCR was performed as described on a panel of mRNA extracted from rat tissue as indicated at the bottom of the gel. After amplification, PCR reactions were size

fractionated on 10% polyacrylamide gels, and stained with SYBR Green I. Images were analyzed using a Molecular Dynamics Storm 860 workstation. The amplified band corresponding to NPFF1 (490 base pairs) is indicated (arrow). RT-PCR indicates a broad distribution of mRNA encoding NPFF1 with highest concentrations found in nervous system structures.

Figure 20

Autoradiograph demonstrating hybridization of radiolabeled rat NPFF1 probe to RNA extracted from rat tissue in a solution hybridization/nuclease protection assay using ^{32}P labeled riboprobe. 2μg of RNA was used in each assay. The single band (arrow) represents mRNA coding for the NPFF1 receptors extracted from the indicated tissue. Highest levels of mRNA coding for NPFF1 are found in: hypothalamus and pituitary gland. The smaller bands representing NPFF1 mRNA from the pituitary, adrenal gland, and ovary (double arrow) may indicate a splice variant present in this tissue. Integrity of RNA was assessed using hybridization to mRNA coding for GAPDH (not shown).

Figure 21

RT-PCR was performed as described on a panel of mRNA extracted from tissue as indicated at the bottom of the gel. After amplification, PCR reactions were size fractionated on 10% polyacrylamide gels, and stained with SYBR Green I. Images were analyzed using a Molecular Dynamics Storm 860 workstation. The amplified band corresponding to NPFF2 receptors (approximately 325 base pairs) is indicated (arrow). RT-PCR indicates a broad distribution of mRNA encoding NPFF2 receptors. The only tissue containing mRNA coding for NPFF2 receptors were HeLa cells and Jurkat cells.

DETAILED DESCRIPTION OF THE INVENTION

Throughout this application, the following standard abbreviations are used to indicate specific nucleotide bases:

A =	adenine
G =	guanine
C =	cytosine
T =	thymine
U =	uracil
M =	adenine or cytosine
R =	adenine or guanine
W =	adenine, thymine, or uracil
S =	cytosine or guanine
Y =	cytosine, thymine, or uracil
K =	guanine, thymine, or uracil
V =	adenine, cytosine, or guanine (not thymine or uracil
H =	adenine, cytosine, thymine, or uracil (not guanine)
D =	adenine, guanine, thymine, or uracil (not cytosine)
B =	cytosine, guanine, thymine, or uracil (not adenine)
N =	adenine, cytosine, guanine, thymine, or uracil (or other modified base such as inosine)
I =	inosine

Furthermore, the term "agonist" is used throughout this application to indicate any peptide or non-peptidyl compound which increases the activity of any of the polypeptides of the subject invention. The term

"antagonist" is used throughout this application to indicate any peptide or non-peptidyl compound which decreases the activity of any of the polypeptides of the subject invention.

The activity of a G-protein coupled receptor such as the polypeptides disclosed herein may be measured using any of a variety of functional assays in which activation of the receptor in question results in an observable change in the level of some second messenger system, including, but not limited to, adenylate cyclase, calcium mobilization, arachidonic acid release, ion channel activity, inositol phospholipid hydrolysis or guanylyl cyclase. Heterologous expression systems utilizing appropriate host cells to express the nucleic acid of the subject invention are used to obtain the desired second messenger coupling. Receptor activity may also be assayed in an oocyte expression system.

It is possible that the mammalian NPFF receptor genes contain introns and furthermore, the possibility exists that additional introns could exist in coding or non-coding regions. In addition, spliced form(s) of mRNA may encode additional amino acids either upstream of the currently defined starting methionine or within the coding region. Further, the existence and use of alternative exons is possible, whereby the mRNA may encode different amino acids within the region comprising the exon. In addition, single amino acid substitutions may arise via the mechanism of RNA editing such that the amino acid sequence of the expressed protein is different than that encoded by the original gene. (Burns et al., 1996; Chu et al., 1996). Such variants may exhibit pharmacologic properties differing from the polypeptide encoded by the

original gene.

This invention provides splice variants of the mammalian NPFF receptors disclosed herein. This invention further provides for alternate translation initiation sites and alternately spliced or edited variants of nucleic acids encoding the mammalian NPFF receptors of this invention.

The nucleic acids of the subject invention also include nucleic acid analogs of the rat and human NPFF receptor genes, wherein the rat NPFF1 receptor gene comprises the nucleic acid sequence shown in Fig. 1 or contained in plasmid pEXJ-rNPFF1 (ATCC Accession No. 203184); the human NPFF1 receptor gene comprises the nucleic acid shown in Figure 4 and contained in plasmid pWE15-hNPFF1 (ATCC Accession No. 203183); the human NPFF2 receptor gene comprises the nucleic acid shown in Figure 7 and contained in plasmid pCDNA3.1-hNPFF2b (ATCC Accession No.203255); or the human NPFF1 receptor gene comprises the nucleic acid shown in Figure 11 and contained in plasmid pcDNA3.1-hNPFF1 (ATCC Accession No.203605). Nucleic acid analogs of the rat and human NPFF receptor genes differ from the rat and human NPFF receptor genes described herein in terms of the identity or location of one or more nucleic acid bases (deletion analogs containing less than all of the nucleic acid bases shown in Figs. 1, 4, 7 or 11 or contained in plasmids pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b or pcDNA3.1-hNPFF1 respectively, substitution analogs wherein one or more nucleic acid bases shown in Figs. 1, 4, 7 or 11 or contained in plasmids pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b or pcDNA3.1-hNPFF1, respectively, are replaced by other nucleic acid bases, and addition analogs, wherein one or more nucleic acid bases are added to a terminal or medial portion of the

nucleic acid sequence) and which encode proteins which share some or all of the properties of the proteins encoded by the nucleic acid sequences shown in Figs. 1, 4, 7 or 11 or contained in plasmids pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, or pcDNA3.1-hNPFF1, respectively. In one embodiment of the present invention, the nucleic acid analog encodes a protein which has an amino acid sequence identical to that shown in Fig. 2, 5 8 or 12 or encoded by the nucleic acid sequence contained in plasmids pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b or pcDNA3.1-hNPFF1, respectively. In another embodiment, the nucleic acid analog encodes a protein having an amino acid sequence which differs from the amino acid sequences shown in Fig. 2, 5, 8 or 12 or encoded by the nucleic acid contained in plasmids pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b or pcDNA3.1-hNPFF1 respectively. In a further embodiment, the protein encoded by the nucleic acid analog has a function which is the same as the function of the receptor proteins having the amino acid sequence shown in Fig. 2, 5, 8 or 12. In another embodiment, the function of the protein encoded by the nucleic acid analog differs from the function of the receptor protein having the amino acid sequence shown in Fig. 2, 5, 8 or 12. In another embodiment, the variation in the nucleic acid sequence occurs within the transmembrane (TM) region of the protein. In a further embodiment, the variation in the nucleic acid sequence occurs outside of the TM region.

This invention provides the above-described isolated nucleic acid, wherein the nucleic acid is DNA. In an embodiment, the DNA is cDNA. In another embodiment, the DNA is genomic DNA. In still another embodiment, the nucleic acid is RNA. Methods for production and manipulation of nucleic acid molecules are well known in

the art.

This invention further provides nucleic acid which is degenerate with respect to the DNA encoding any of the polypeptides described herein. In an embodiment, the nucleic acid comprises a nucleotide sequence which is degenerate with respect to the nucletide sequence shown in Figures 1 (SEQ I.D. No. 1), 4 (SEQ I.D. No. 3), 7 (SEQ I.D. No. 5) or 11 (SEQ I.D. No. 7) or the nucleotide sequence contained in the plasmids pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b or pcDNA3.1-hNPFF1, respectively, that is, a nucleotide sequence which is translated into the same amino acid sequence.

This invention also encompasses DNAs and cDNAs which encode amino acid sequences which differ from those of the polypeptides of this invention, but which should not produce phenotypic changes. Alternately, this invention also encompasses DNAs, cDNAs, and RNAs which hybridize to the DNA, cDNA, and RNA of the subject invention. Hybridization methods are well known to those of skill in the art.

The nucleic acids of the subject invention also include nucleic acid molecules coding for polypeptide analogs, fragments or derivatives of antigenic polypeptides which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs wherein one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties

of naturally-occurring forms. These molecules include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors. The creation of polypeptide analogs is well known to those of skill in the art (R.F. Spurney et al. (1997); Fong, T.M. et al. (1995); Underwood, D.J. et al. (1994); Graziano, M.P. et al. (1996); Guam X.M. et al. (1995)).

The modified polypeptides of this invention may be transfected into cells either transiently or stably using methods well-known in the art, examples of which are disclosed herein. This invention also provides for binding assays using the modified polypeptides, in which the polypeptide is expressed either transiently or in stable cell lines. This invention further provides a compound identified using a modified polypeptide in a binding assay such as the binding assays described herein.

The nucleic acids described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide and as products for the large scale synthesis of the polypeptides by a variety of recombinant techniques. The nucleic acid molecule is useful for generating new cloning and expression vectors, transformed and transfected prokaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of the polypeptide and related products.

This invention provides an isolated nucleic acid encoding

a mammalian NPFF receptor. In one embodiment, the nucleic acid is DNA. In another embodiment, the DNA is cDNA. In another embodiment, the DNA is genomic DNA. In another embodiment, the nucleic acid is RNA.

In one embodiment, the mammalian NPFF receptor is a NPFF1 receptor. In a further embodiment, the mammalian NPFF1 receptor is a rat NPFF1 receptor. In another embodiment, the mammalian NPFF1 receptor is a human NPFF1 receptor. In a further embodiment, the mammalian NPFF receptor is a NPFF2 receptor. In one embodiment, the mammalian NPFF2 receptor is a human NPFF2 receptor.

This invention also provides an isolated nucleic acid encoding species homologs of the NPFF receptors encoded by the nucleic acid sequence shown in Figs. 1 (Seq. I.D. No. 1), 4 (Seq. I.D. No. 3), 7 (Seq. I.D. No. 5), or 11 (Seq. I.D. No. 7) or encoded by the plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b or pcDNA3.1-hNPFF1, respectively. In one embodiment, the nucleic acid encodes a mammalian NPFF receptor homolog which has substantially the same amino acid sequence as does the NPFF receptor encoded by the plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b or pcDNA3.1-hNPFF1. In another embodiment, the nucleic acid encodes a mammalian NPFF receptor homolog which has above 65% amino acid identity to the NPFF receptor encoded by the plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, or pcDNA3.1-hNPFF1; preferably above 75% amino acid identity to the NPFF receptor encoded by the plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, or pcDNA3.1-hNPFF1; more preferably above 85% amino acid identity to the NPFF receptor encoded by the plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, or pcDNA3.1-hNPFF1; most preferably above 95% amino acid identity to the NPFF

receptor encoded by the plasmid pEXJ-rNPFF1, PWE15-hNPFF1, pCDNA3.1-hNPFF2b, or pcDNA3.1-hNPFF1. In another embodiment, the mammalian NPFF receptor homolog has above 70% nucleic acid identity to the NPFF receptor gene contained in plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, or pcDNA3.1-hNPFF1; preferably above 80% nucleic acid identity to the NPFF receptor gene contained in the plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, or pcDNA3.1-hNPFF1; more preferably above 90% nucleic acid identity to the NPFF receptor gene contained in the plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, or pcDNA3.1-hNPFF1. Examples of methods for isolating and purifying species homologs are described elsewhere (e.g., U.S. Patent No. 5,602,024, WO94/14957, WO97/26853, WO98/15570).

In separate embodiments of the present invention, the nucleic acid encodes a NPFF receptor which has an amino acid sequence identical to that encoded by the plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b, or pcDNA3.1-hNPFF1. In further embodiments, the NPFF receptor has a sequence substantially the same as the amino acid sequence shown in Figure 2 (Seq. I.D. No. 2), Figure 5 (Seq. I.D. No. 4), Figure 8 (Seq. I.D. No. 6) or Figure 12 (Seq. I.D. No. 8). In other embodiments, the NPFF receptor has an amino acid sequence identical to the amino acid sequence shown in Figure 2 (Seq. I.D. No. 2), Figure 5 (Seq. I.D. No. 4), Figure 8 (Seq. I.D. No. 6) or Figure 12 (Seq. I.D. No. 8).

This invention provides an isolated nucleic acid encoding a modified mammalian NPFF receptor, which differs from a mammalian NPFF receptor by having an amino acid(s) deletion, replacement, or addition in the third

intracellular domain.

This invention provides a nucleic acid encoding a mammalian NPFF receptor, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figure 1 (Seq. I.D. No. 1) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its complement. This invention further provides a nucleic acid encoding a mammalian NPFF receptor, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figure 4 (Seq. I.D. No. 3) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its complement. This invention also provides a nucleic acid encoding a mammalian NPFF receptor, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figure 7 (Seq. I.D. No. 5) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its complement.

This invention further provides a nucleic acid encoding a

mammalian NPFF receptor, wherein the nucleic acid (a) hybridizes to a nucleic acid having the defined sequence shown in Figure 11 (Seq. I.D. No. 7) under low stringency conditions or a sequence complementary thereto and (b) is further characterized by its ability to cause a change in the pH of a culture of CHO cells when a NPFF peptide is added to the culture and the CHO cells express the nucleic acid which hybridized to the nucleic acid having the defined sequence or its complement.

In one embodiment, the mammalian NPFF receptor is a rat NPFF1 receptor. In another embodiment, the mammalian NPFF receptor is a human NPFF1 receptor. In a further embodiment, the mammalian NPFF receptor is a human NPFF2 receptor. For purpose of the invention hybridization under low stringency conditions means hybridization performed at 40°C in a hybridization buffer containing 25% formamide, 5X SCC, 7mM Tris, 1X Denhardt's, 25μl/ml salmon sperm DNA. Wash at 40°C in 0.1X SCC, 0.1% SDS. Changes in pH are measured through microphysiometric measurement of receptor mediated extracellular acidification rates. Because cellular metabolism is intricately involved in a broad range of cellular events (including receptor activation of multiple messenger pathways), the use of microphysiometric measurements of cell metabolism can in principle provide a generic assay of cellular activity arising from the activation of any receptor regardless of the specifics of the receptor's signaling pathway. General guidelines for transient receptor expression, cell preparation and microphysiometric recording are described elsewhere (Salon, J.A. and Owicki, J.A., 1996). Receptors and/or control vectors are transiently expressed in CHO-K1 cells, by liposome mediated transfection according to the manufacturers recommendations (LipofectAMINE, GibcoBRL,

Gaithersburg, MD), and maintained in Ham's F-12 complete (10% serum). A total of 10μg of DNA is used to transfect each 75cm^2 flask which had been split 24 hours prior to the transfection and judged to be 70-80% confluent at the time of transfection. 24 hours post transfection, the cells are harvested and 3 x 10^5 cells seeded into microphysiometer capsules. Cells are allowed to attach to the capsule membrane for an additional 24 hours; during the last 16 hours, the cells are switched to serum-free F-12 complete to minimize ill-defined metabolic stimulation caused by assorted serum factors. On the day of the experiment the cell capsules are transferred to the microphysiometer and allowed to equilibrate in recording media (low buffer RPMI 1640, no bicarbonate, no serum (Molecular Devices Corporation, Sunnyvale, CA) containing 0.1% fatty acid free BSA), during which a baseline measurement of basal metabolic activity is established. A standard recording protocol specifies a 100μl/min flow rate, with a 2 min total pump cycle which includes a 30 sec flow interruption during which the acidification rate measurement is taken. Ligand challenges involve a 1 min 20 sec exposure to the sample just prior to the first post challenge rate measurement being taken, followed by two additional pump cycles for a total of 5 min 20 sec sample exposure. Typically, drugs in a primary screen are presented to the cells at 10μM final concentration. Ligand samples are then washed out and the acidification rates reported are expressed as a percentage increase of the peak response over the baseline rate observed just prior to challenge. Endogenous NPFF peptides include rat NPFF (FLFQPQRF-NH2) and rat A18Fa (AGEGLSSPFWSLAAPQRF-NH2).

This invention provides a purified mammalian NPFF receptor

protein. In one embodiment, the purified mammalian NPFF receptor protein is a human NPFF1 receptor protein. In another embodiment, the purified mammalian NPFF receptor protein is a rat NPFF1 receptor protein. In a further embodiment, the purified mammalian NPFF receptor protein is a human NPFF2 receptor protein.

This invention provides a vector comprising nucleic acid encoding a mammalian NPFF receptor. In one embodiment, the mammalian NPFF receptor protein is a NPFF1 receptor protein. In another embodiment of the present invention the mammalian NPFF receptor protein is a NPFF2 receptor protein. In one embodiment, the mammalian NPFF receptor is a rat NPFF1 receptor. In another embodiment, the mammalian NPFF receptor is a human NPFF1 receptor. In a further embodiment, the mammalian NPFF receptor is a human NPFF2 receptor.

In an embodiment, the vector is adapted for expression in a cell which comprises the regulatory elements necessary for expression of the nucleic acid in the cell operatively linked to the nucleic acid encoding the mammalian NPFF receptor as to permit expression thereof. In separate embodiments, the cell is a bacterial cell, an amphibian cell, a yeast cell, an insect cell or a mammalian cell. In another embodiment, the vector is a baculovirus. In one embodiment, the vector is a plasmid.

This invention provides a plasmid designated pEXJ-rNPFF1 (ATCC Accession No. 203184). This plasmid comprises the regulatory elements necessary for expression of DNA in a mammalian cell operatively linked to DNA encoding the mammalian NPFF1 receptor so as to permit expression thereof. This invention also provides a plasmid

designated pWE15-hNPFF1 (ATCC Accession No. 203183). This invention further provides a plasmid designated pCDNA3.1-hNPFF2b (ATCC Accession No. 203255). This invention additionally provides a plasmid designated pcDNA3.1-hNPFF1 (ATCC Accession No. 203605).

These plasmids (pEXJ-rNPFF1 and pWE15-hNPFF1) were deposited on September 9, 1998, with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia 20110-2209, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and were accorded ATCC Accession Nos. 203184 and 203183, respectively. Plasmid pCDNA3.1-hNPFF2b was deposited on September 22, 1998, with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia 20110-2209, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and was accorded ATCC Accession No. 203255. Plasmid pcDNA3.1-hNPFF1 was deposited on January 21, 1999, with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia 20110-2209, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and was accorded ATCC Accession No. 203605.

This invention further provides for any vector or plasmid which comprises modified untranslated sequences, which are beneficial for expression in desired host cells or for use in binding or functional assays. For example, a vector or plasmid with untranslated sequences of varying lengths may express differing amounts of the polypeptide depending

upon the host cell used. In an embodiment, the vector or plasmid comprises the coding sequence of the polypeptide and the regulatory elements necessary for expression in the host cell.

This invention provides a cell comprising a vector comprising a nucleic acid encoding the mammalian NPFF receptor. In an embodiment, the cell is a non-mammalian cell. In a further embodiment, the non-mammalian cell is a Xenopus oocyte cell or a Xenopus melanophore cell. In another embodment, the cell is a mammalian cell. In a further embodiment, the mammalian cell is a COS-7 cell, a 293 human embryonic kidney cell, a NIH-3T3 cell, a LM(tk-) cell, a mouse Y1 cell, or a CHO cell.

This invention provides an insect cell comprising a vector adapted for expression in an insect cell which comprises a nucleic acid encoding a mammalian NPFF receptor. In another embodiment, the insect cell is an Sf9 cell, an Sf21 cell or a Trichoplusia ni 5B1-4 (HighFive) cell.

This invention provides a membrane preparation isolated from any one of the cells described above.

This invention provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within one of the two strands of the nucleic acid encoding the mammalian NPFF receptor and are contained in plasmid pEXJ-rNPFF1, plasmid pWE15-hNPFF1, pCDNA3.1-hNPFF2b or pcDNA3.1-hNPFF1. This invention also provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a

nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 1 (Seq. I.D. No. 1) or (b) the reverse complement thereto. This invention also provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 4 (Seq. I.D. No. 3) or (b) the reverse complement thereto. This invention also provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 7 (Seq. I.D. No. 5) or (b) the reverse complement thereto. This invention also provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian NPFF receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 11 (Seq. I.D. No. 7) or (b) the reverse complement thereto. In one embodiment, the nucleic acid is DNA. In another embodiment, the nucleic acid is RNA.

As used herein, the phrase "specifically hybridizing" means the ability of a nucleic acid molecule to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen bonding between complementary base pairs.

Nucleic acid probe technology is well known to those

skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable label, such as a radioisotope or flourescent dye, to facilitate detection of the probe. DNA probe molecules may be produced by insertion of a DNA molecule which encodes the polypeptides of this invention into suitable vectors, such as plasmids or bacteriophages, followed by transforming into suitable bacterial host cells, replication in the transformed bacterial host cells and harvesting of the DNA probes, using methods well known in the art. Alternatively, probes may be generated chemically from DNA synthesizers.

RNA probes may be generated by inserting the DNA molecule which encodes the polypeptides of this invention downstream of a bacteriophage promoter such as T3, T7, or SP6. Large amounts of RNA probe may be produced by incubating the labeled nucleotides with the linearized fragment where it contains an upstream promoter in the presence of the appropriate RNA polymerase.

This invention provides an antisense oligonucleotide having a sequence capable of specifically hybridizing to RNA encoding a mammalian NPFF receptor, so as to prevent translation of the RNA. This invention also provides an antisense oligonucleotide having a sequence capable of specifically hybridizing to genomic DNA encoding a mammalian NPFF receptor, so as to prevent translation of the genomic DNA. In one embodiment, the oligonucleotide comprises chemically modified nucleotides or nucleotide analogues.

This invention provides an antibody capable of binding to a mammalian NPFF receptor encoded by a nucleic acid

encoding a mammalian NPFF receptor. In one embodiment, the mammalian NPFF receptor is a rat NPFF1 receptor. In another embodiment, the mammalian NPFF receptor is a human NPFF1 receptor. In a further embodiment, the mammalian NPFF receptor is a human NPFF2 receptor. This invention also provides an agent capable of competitively inhibiting the binding of the antibody to a mammalian NPFF receptor. In one embodiment, the antibody is a monoclonal antibody or antisera.

This invention provides a pharmaceutical composition comprising (a) an amount of the oligonucleotide capable of passing through a cell membrane and effective to reduce expression of a mammalian NPFF receptor and (b) a pharmaceutically acceptable carrier capable of passing through the cell membrane. In an embodiment, the oligonucleotide is coupled to a substance which inactivates mRNA. In a further embodiment, the substance which inactivates mRNA is a ribozyme. In another embodiment, the pharmaceutically acceptable carrier comprises a structure which binds to a mammalian NPFF receptor on a cell capable of being taken up by the cells after binding to the structure. In a further embodiment, the pharmaceutically acceptable carrier is capable of binding to a mammalian NPFF receptor which is specific for a selected cell type.

This invention provides a pharmaceutical composition which comprises an amount of an antibody effective to block binding of a ligand to a human NPFF receptor and a pharmaceutically acceptable carrier.

As used herein, the phrase "pharmaceutically acceptable carrier" means any of the standard pharmceutically

acceptable carriers. Examples include, but are not limited to, phosphate buffered saline, physiological saline, water, and emulsions, such as oil/water emulsions.

This invention provides a transgenic, nonhuman mammal expressing DNA encoding a mammalian NPFF receptor. This invention also provides a transgenic, nonhuman mammal comprising a homologous recombination knockout of the native mammalian NPFF receptor. This invention further provides a transgenic, nonhuman mammal whose genome comprises antisense DNA complementary to the DNA encoding a mammalian NPFF receptor so placed within the genome as to be transcribed into antisense mRNA which is complementary to mRNA encoding the mammalian NPFF receptor and which hybridizes to mRNA encoding the mammalian NPFF receptor, thereby reducing its translation. In an embodiment, the DNA encoding the mammalian NPFF receptor additionally comprises an inducible promoter. In another embodiment, the DNA encoding the mammalian NPFF receptor additionally comprises tissue specific regulatory elements. In a further embodiment, the transgenic, nonhuman mammal is a mouse.

Animal model systems which elucidate the physiological and behavioral roles of the polypeptides of this invention are produced by creating transgenic animals in which the activity of the polypeptide is either increased or decreased, or the amino acid sequence of the expressed polypeptide is altered, by a variety of techniques. Examples of these techniques include, but are not limited to: 1) Insertion of normal or mutant versions of DNA encoding the polypeptide, by microinjection, electroporation, retroviral transfection or other means well known to those in the art, into appropriate

fertilized embryos in order to produce a transgenic animal or 2) Homologous recombination of mutant or normal, human or animal versions of these genes with the native gene locus in transgenic animals to alter the regulation of expression or the structure of these polypeptide sequences. The technique of homologous recombination is well known in the art. It replaces the native gene with the inserted gene and so is useful for producing an animal that cannot express native polypeptides but does express, for example, an inserted mutant polypeptide, which has replaced the native polypeptide in the animal's genome by recombination, resulting in underexpression of the transporter. Microinjection adds genes to the genome, but does not remove them, and so is useful for producing an animal which expresses its own and added polypeptides, resulting in overexpression of the polypeptides.

One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as M2 medium. DNA or cDNA encoding a polypeptide of this invention is purified from a vector by methods well known in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the trans-gene. Alternatively, or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the trans-gene. The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a pipet puller) and the egg to be injected is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is

injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (a mouse stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only method for inserting DNA into the egg cell, and is used here only for exemplary purposes.

This invention provides a process for identifying a chemical compound which specifically binds to a mammalian NPFF receptor which comprises contacting cells containing DNA encoding and expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the mammalian NPFF receptor. This invention also provides a process for identifying a chemical compound which specifically binds to a mammalian NPFF receptor which comprises contacting a membrane fraction from a cell extract of cells containing DNA encoding and expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the mammalian NPFF receptor. In one embodiment, the NPFF receptor is a NPFF1 receptor. In a further embodiment, the mammalian NPFF1 receptor is a rat NPFF1 receptor. In another embodiment, the mammalian NPFF1 receptor is a human NPFF1 receptor. In one embodiment, the mammalian NPFF receptor is a NPFF2 receptor. In a further embodiment, the mammalian NPFF2 receptor is a human NPFF2 receptor. In another

embodiment, the mammalian NPFF receptor has substantially the same amino acid sequence as the NPFF receptor encoded by plasmid pEXJ-rNPFF1, plasmid pWE15-hNPFF1, plasmid pCDNA3.1-hNPFF2b or plasmid pcDNA3.1-hNPFF1. In a further embodiment, the mammalian NPFF receptor has substantially the same amino acid sequence as that shown in Figure 2 (Seq. I.D. No. 2), Figure 5 (Seq. I.D. No. 4), Figure 8 (Seq. I.D. No. 6), or Figure 12 (Seq. I.D. No. 8). In another embodiment, the mammalian NPFF receptor has the amino acid sequence shown in Figure 2 (Seq. I.D. No. 2), Figure 5 (Seq. I.D. No. 4) Figure 8 (Seq. I.D. No. 6), or Figure 12 (Seq. I.D. No. 8). In one embodiment, the compound is not previously known to bind to a mammalian NPFF receptor. This invention further provides a compound identified by the above-described processes.

In one embodiment of the above-described processes, the cell is an insect cell. In another embodiment, the cell is a mammalian cell. In a further embodiment, the cell is nonneuronal in origin. In a further embodiment, the nonneuronal cell is a COS-7 cell, 293 human embryonic kidney cell, a CHO cell, a NIH-3T3 cell, a mouse Y1 cell, or a LM(tk-) cell. In an embodiment, the compound is a compound not previously known to bind to a mammalian NPFF receptor. This invention also provides a compound identified by the above-described process.

This invention provides a process involving competitive binding for identifying a chemical compound which specifically binds to a mammalian NPFF receptor which comprises separately contacting cells expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with both the chemical compound and a second chemical

compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the mammalian NPFF receptor, a decrease in the binding of the second chemical compound to the mammalian NPFF receptor in the presence of the chemical compound indicating that the chemical compound binds to the mammalian NPFF receptor.

This invention also provides a process involving competitive binding for identifying a chemical compound which specifically binds to a mammalian NPFF receptor which comprises separately contacting a membrane preparation from cells expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the mammalian NPFF receptor, a decrease in the binding of the second chemical compound to the mammalian NPFF receptor in the presence of the chemical compound indicating that the chemical compound binds to the mammalian NPFF receptor.

In one embodiment, the mammalian NPFF receptor is a NPFF1 receptor. In a further embodiment, the mammalian NPFF1 receptor is a rat NPFF1 receptor. In another embodiment, the mammalian NPFF1 receptor is a human NPFF1 receptor. In another embodiment, the mammalian NPFF receptor is a NPFF2 receptor. In a further embodiment, the NPFF2 receptor is a human NPFF2 receptor. In another embodiment, the mammalian NPFF receptor has substantially

the same amino acid sequence as the NPFF receptor encoded by plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b or pcDNA3.1-hNPFF1. In a further embodiment, the mammalian NPFF receptor has substantially the same amino acid sequence as that shown in Figure 2 (Seq. I.D. No. 2), Figure 5 (Seq. I.D. No. 4), Figure 8 (Seq. I.D. No. 6) or Figure 12 (Seq. I.D. No. 8). In another embodiment, the mammalian NPFF receptor has the amino acid sequence shown in Figure 2 (Seq. I.D. No. 2), Figure 5 (Seq. I.D. No. 4), Figure 8 (Seq. I.D. No. 6) or Figure 12 (Seq. I.D. No. 8).

In one embodiment, the cell is an insect cell. In another embodiment, the cell is a mammalian cell. In a further embodiment, the cell is nonneuronal in origin. In another embodiment, the nonneuronal cell is a COS-7 cell, 293 human embryonic kidney cell, a CHO cell, a NIH-3T3 cell, a mouse Y1 cell, or a LM(tk-) cell. In one embodiment, the compound is not previously known to bind to a mammalian NPFF receptor.

This invention provides a compound identified by the above-described processes.

This invention provides a method of screening a plurality of chemical compounds not known to bind to a mammalian NPFF receptor to identify a compound which specifically binds to the mammalian NPFF receptor, which comprises (a) contacting cells transfected with and expressing DNA encoding the mammalian NPFF receptor with a compound known to bind specifically to the mammalian NPFF receptor; (b) contacting the preparation of step (a) with the plurality of compounds not known to bind specifically to the mammalian NPFF receptor, under conditions permitting binding of compounds known to bind the mammalian NPFF

receptor; (c) determining whether the binding of the compound known to bind to the mammalian NPFF receptor is reduced in the presence of the compounds within the plurality of compounds, relative to the binding of the compound in the absence of the plurality of compounds; and if so (d) separately determining the binding to the mammalian NPFF receptor of compounds included in the plurality of compounds, so as to thereby identify the compound which specifically binds to the mammalian NPFF receptor.

This invention provides a method of screening a plurality of chemical compounds not known to bind to a mammalian NPFF receptor to identify a compound which specifically binds to the mammalian NPFF receptor, which comprises (a) contacting a membrane preparation from cells transfected with and expressing DNA encoding the mammalian NPFF receptor with the plurality of compounds not known to bind specifically to the mammalian NPFF receptor under conditions permitting binding of compounds known to bind to the mammalian NPFF receptor; (b) determining whether the binding of a compound known to bind to the mammalian NPFF receptor is reduced in the presence of any compound within the plurality of compounds, relative to the binding of the compound in the absence of the plurality of compounds; and if so (c) separately determining the binding to the mammalian NPFF receptor of compounds included in the plurality of compounds, so as to thereby identify the compound which specifically binds to the mammalian NPFF receptor.

This invention provides a method of screening a plurality of chemical compounds not known to bind to a mammalian NPFF receptor to identify a compound which specifically

binds to the mammalian NPFF receptor, which comprises (a) contacting a membrane preparation from cells transfected with and expressing the mammalian NPFF receptor with a compound known to bind specifically to the mammalian NPFF receptor; (b) contacting the preparation of step (a) with the plurality of compounds not known to bind specifically to the mammalian NPFF receptor, under conditions permitting binding of compounds known to bind the mammalian NPFF receptor; (c) determining whether the binding of the compound known to bind to the mammalian NPFF receptor is reduced in the presence of the compounds within the plurality of compounds, relative to the binding of the compound in the absence of the plurality of compounds; and if so (d) separately determining the binding to the mammalian NPFF receptor of compounds included in the plurality of compounds, so as to thereby identify the compound which specifically binds to the mammalian NPFF receptor.

In one embodiment of the above-described methods, the mammalian NPFF receptor is a NPFF1 receptor. In a further embodiment, the mammalian NPFF1 receptor is a rat NPFF1 receptor. In another embodiment, the mammalian NPFF1 receptor is a human NPFF1 receptor. In another embodiment, the mammalian NPFF receptor is a NPFF2 receptor. In a further embodiment the NPFF2 receptor is a human NPFF2 receptor. In another embodiment, the cell is a mammalian cell. In a further embodiment, the mammalian cell is non-neuronal in origin. In another embodiment, the non-neuronal cell is a COS-7 cell, a 293 human embryonic kidney cell, a LM(tk-) cell, a CHO cell, a mouse Y1 cell, or an NIH-3T3 cell.

This invention also provides a method of detecting

expression of a mammalian NPFF receptor by detecting the presence of mRNA coding for the mammalian NPFF receptor which comprises obtaining total mRNA from the cell and contacting the mRNA so obtained from a nucleic acid probe under hybridizing conditions, detecting the presence of mRNA hybridizing to the probe, and thereby detecting the expression of the mammalian NPFF receptor by the cell.

This invention further provides a method of detecting the presence of a mammalian NPFF receptor on the surface of a cell which comprises contacting the cell with an antibody under conditions permitting binding of the antibody to the receptor, detecting the presence of the antibody bound to the cell, and thereby detecting the presence of the mammalian NPFF receptor on the surface of the cell.

This invention provides a method of determining the physiological effects of varying levels of activity of mammalian NPFF receptors which comprises producing a transgenic, nonhuman mammal whose levels of mammalian NPFF receptor activity are varied by use of an inducible promoter which regulates mammalian NPFF receptor expression.

This invention also provides a method of determining the physiological effects of varying levels of activity of mammalian NPFF receptors which comprises producing a panel of transgenic, nonhuman mammals each expressing a different amount of mammalian NPFF receptor.

This invention provides a method for identifying an antagonist capable of alleviating an abnormality wherein the abnormality is alleviated by decreasing the activity of a mammalian NPFF receptor comprising administering a

compound to a transgenic, nonhuman mammal, and determining whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic, nonhuman mammal as a result of overactivity of a mammalian NPFF receptor, the alleviation of the abnormality identifying the compound as an antagonist. This invention also provides an antagonist identified by the above-described method. This invention further provides a pharmaceutical composition comprising an antagonist identified by the above-described method and a pharmaceutically acceptable carrier. This invention provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by decreasing the activity of a mammalian NPFF receptor which comprises administering to the subject an effective amount of this pharmaceutical composition, thereby treating the abnormality.

This invention provides a method for identifying an agonist capable of alleviating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a mammalian NPFF receptor comprising administering a compound to transgenic, nonhuman mammal, and determining whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic, nonhuman mammal, the alleviation of the abnormality identifying the compound as an agonist. This invention also provides an agonist identified by the above-described method. This invention further provides a pharmaceutical composition comprising an agonist identified by the above-described method and a pharmaceutically acceptable carrier. This invention further provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by

increasing the activity of a mammalian NPFF receptor which comprises administering to the subject an effective amount of this pharmaceutical composition, thereby treating the abnormality.

This invention provides a method for diagnosing a predisposition to a disorder associated with the activity of a specific mammalian allele which comprises: (a) obtaining DNA of subjects suffering from the disorder; (b) performing a restriction digest of the DNA with a panel of restriction enzymes; (c) electrophoretically separating the resulting DNA fragments on a sizing gel; (d) contacting the resulting gel with a nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a mammalian NPFF receptor and labeled with a detectable marker; (e) detecting labeled bands which have hybridized to the DNA encoding a mammalian NPFF receptor labeled with a detectable marker to create a unique band pattern specific to the DNA of subjects suffering from the disorder; (f) preparing DNA obtained for diagnosis by steps (a)-(e); and (g) comparing the unique band pattern specific to the DNA of subjects suffering from the disorder from step (e) and the DNA obtained for diagnosis from step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to the disorder if the patterns are the same. In one embodiment, a disorder associated with the activity of a specific mammalian allele is diagnosed.

This invention provides a method of preparing the purified mammalian NPFF receptor which comprises: (a) inducing cells to express the mammalian NPFF receptor; (b) recovering the mammalian NPFF receptor from the induced

cells; and (c) purifying the mammalian NPFF receptor so recovered.

This invention provides a method of preparing the purified mammalian NPFF receptor which comprises: (a) inserting nucleic acid encoding the mammalian NPFF receptor in a suitable vector; (b) introducing the resulting vector in a suitable host cell; (c) placing the resulting cell in suitable condition permitting the production of the isolated mammalian NPFF receptor; (d) recovering the mammalian NPFF receptor produced by the resulting cell; and (e) purifying the mammalian NPFF receptor so recovered.

This invention provides a process for determining whether a chemical compound is a mammalian NPFF receptor agonist which comprises contacting cells transfected with and expressing DNA encoding the mammalian NPFF receptor with the compound under conditions permitting the activation of the mammalian NPFF receptor, and detecting an increase in mammalian NPFF receptor activity, so as to thereby determine whether the compound is a mammalian NPFF receptor agonist. This invention also provides a process for determining whether a chemical compound is a mammalian NPFF1 receptor antagonist which comprises contacting cells transfected with and expressing DNA encoding the mammalian NPFF receptor with the compound in the presence of a known mammalian NPFF receptor agonist, under conditions permitting the activation of the mammalian NPFF receptor, and detecting a decrease in mammalian NPFF receptor activity, so as to thereby determine whether the compound is a mammalian NPFF receptor antagonist. In one embodiment, the mammalian NPFF receptor is a NPFF1 receptor. In a further embodiment, the mammalian NPFF1

receptor is a rat NPFF1 receptor. In another embodiment, the mammalian NPFF1 receptor is a human NPFF1 receptor. In one embodiment, the mammalian NPFF receptor is a NPFF2 receptor. In a further embodiment, the mammalian NPFF2 receptor is a human NPFF2 receptor.

This invention further provides a pharmaceutical composition which comprises an amount of a mammalian NPFF receptor agonist determined by the above-described process effective to increase activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier. In one embodiment, the mammalian NPFF receptor agonist is not previously known.

This invention provides a pharmaceutical composition which comprises an amount of a mammalian NPFF receptor antagonist determined by the above-described process effective to reduce activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier. In one embodiment, the mammalian NPFF receptor antagonist is not previously known.

This invention provides a process for determining whether a chemical compound specifically binds to and activates a mammalian NPFF receptor, which comprises contacting cells producing a second messenger response and expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with the chemical compound under conditions suitable for activation of the mammalian NPFF receptor, and measuring the second messenger response in the presence and in the absence of the chemical compound, a change in the second messenger response in the presence of the chemical compound indicating that the compound

activates the mammalian NPFF receptor. In one embodiment, the second messenger response comprises chloride channel activation and the change in second messenger is an increase in the level of inward chloride current.

This invention also provides a process for determining whether a chemical compound specifically binds to and inhibits activation of a mammalian NPFF receptor, which comprises separately contacting cells producing a second messenger response and expressing on their cell surface the mammalian NPFF receptor, wherein such cells do not normally express the mammalian NPFF receptor, with both the chemical compound and a second chemical compound known to activate the mammalian NPFF receptor, and with only the second chemical compound, under conditions suitable for activation of the mammalian NPFF receptor, and measuring the second messenger response in the presence of only the second chemical compound and in the presence of both the second chemical compound and the chemical compound, a smaller change in the second messenger response in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound indicating that the chemical compound inhibits activation of the mammalian NPFF receptor. In one embodiment, the second messenger response comprises chloride channel activation and the change in second messenger response is a smaller increase in the level of inward chloride current in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound. This invention also provides the above-described processes performed with membrane preparations from cells producing a second messenger response and transfected with and expressing the mammalian NPFF receptor.

In one embodiment of the above-described processes, the mammalian NPFF receptor is a NPFF1 receptor. In a further embodiment, the mammalian NPFF1 receptor is a rat NPFF1 receptor. In another embodiment, the mammalian NPFF1 receptor is a human NPFF1 receptor. In another embodiment, the mammalian NPFF receptor is a NPFF2 receptor. In a further embodiment, the mammalian NPFF2 receptor is a human NPFF2 receptor. In another embodiment, the mammalian NPFF receptor has substantially the same amino acid sequence as encoded by the plasmid pEXJ-rNPFF1, pWE15-hNPFF1, pCDNA3.1-hNPFF2b or pcDNA3.1-hNPFF1. In a further embodiment, the mammalian NPFF receptor has substantially the same amino acid sequence as that shown in Figure 2 (Seq. I.D. No. 2), Figure 5 (Seq. I.D. No. 4), Figure 8 (Seq. I.D. No. 6) or Figure 12 (Seq. I.D. No. 8). In another embodiment, the mammalian NPFF receptor has an amino acid sequence identical to the amino acid sequence shown in Figure 2 (Seq. I.D. No. 2), Figure 5 (Seq. I.D. No. 4), Figure 8 (Seq. I.D. No. 6) or Figure 12 (Seq. I.D. No. 8). In an embodiment, the cell is an insect cell. In a further embodiment, the cell is a mammalian cell. In a still further embodiment, the mammalian cell is nonneuronal in origin. In another embodiment, the nonneuronal cell is a COS-7 cell, CHO cell, 293 human embryonic kidney cell, NIH-3T3 cell or LM(tk-) cell. In an embodiment, the compound is not previously known to bind to a mammalian NPFF receptor. This invention also provides a compound determined by the above-described processes.

This invention also provides a pharmaceutical composition which comprises an amount of a mammalian NPFF receptor agonist determined by the above-described processes effective to increase activity of a mammalian NPFF

receptor and a pharmaceutically acceptable carrier. In one embodiment, the mammalian NPFF receptor agonist is not previously known.

This invention further provides a pharmaceutical composition which comprises an amount of a mammalian NPFF receptor antagonist determined by the above-described processes effective to reduce activity of a mammalian NPFF receptor and a pharmaceutically acceptable carrier. In one embodiment, the mammalian NPFF receptor antagonist is not previously known.

This invention provides a method of screening a plurality of chemical compounds not known to activate a mammalian NPFF receptor to identify a compound which activates the mammalian NPFF receptor which comprises: (a) contacting cells transfected with and expressing the mammalian NPFF receptor with the plurality of compounds not known to activate the mammalian NPFF receptor, under conditions permitting activation of the mammalian NPFF receptor; (b) determining whether the activity of the mammalian NPFF receptor is increased in the presence of the compounds; and if so (c) separately determining whether the activation of the mammalian NPFF receptor is increased by each compound included in the plurality of compounds, so as to thereby identify the compound which activates the mammalian NPFF receptor. In one embodiment, the mammalian NPFF receptor is a human NPFF receptor. In a further embodiment the human NPFF receptor is a human NPFF1 receptor or a human NPFF2 receptor.

This invention provides a method of screening a plurality of chemical compounds not known to inhibit the activation of a mammalian NPFF receptor to identify a compound which

inhibits the activation of the mammalian NPFF receptor, which comprises: (a) contacting cells transfected with and expressing the mammalian NPFF receptor with the plurality of compounds in the presence of a known mammalian NPFF receptor agonist, under conditions permitting activation of the mammalian NPFF receptor; (b) determining whether the activation of the mammalian NPFF receptor is reduced in the presence of the plurality of compounds, relative to the activation of the mammalian NPFF receptor in the absence of the plurality of compounds; and if so (c) separately determining the inhibition of activation of the mammalian NPFF receptor for each compound included in the plurality of compounds, so as to thereby identify the compound which inhibits the activation of the mammalian NPFF receptor. In one embodiment, the mammalian NPFF receptor is a NPFF1 receptor. In a further embodiment, the mammalian NPFF1 receptor is a rat NPFF1 receptor. In another embodiment, the NPFF1 receptor is a human NPFF1 receptor. In another embodiment, the mammalian NPFF receptor is a NPFF2 receptor. In a further embodiment, the NPFF2 receptor is a human NPFF2 receptor.

In one embodiment of the above-described methods, the cell is a mammalian cell. In another embodiment, the mammalian cell is non-neuronal in origin. In a further embodiment, the non-neuronal cell is a COS-7 cell, a 293 human embryonic kidney cell, a LM(tk-) cell or an NIH-3T3 cell.

This invention provides a pharmaceutical composition comprising a compound identified by the above-described methods effective to increase mammalian NPFF receptor activity and a pharmaceutically acceptable carrier.

This invention also provides a pharmaceutical composition

comprising a compound identified by the above-described methods effective to decrease mammalian NPFF receptor activity and a pharmaceutically acceptable carrier.

This invention further provides a method of measuring polypeptide activation in an oocyte expression system such as a Xenopus oocyte expression system or melanophore. In an embodiment, polypeptide activation is determined by measurement of ion channel activity. In another embodiment, polypeptide activation is measured by aequerin luminescence.

Expression of genes in Xenopus oocytes is well known in the art (Coleman, A., 1984; Masu, Y.,et al., 1994) and is performed using microinjection of native mRNA or in vitro synthesized mRNA into frog oocytes. The preparation of in vitro synthesized mRNA can be performed by various standard techniques (Sambrook, et al. 1989) including using T7 polymerase with the mCAP RNA mapping kit (Stratagene).

This invention provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a mammalian NPFF receptor which comprises administering to the subject an amount of a compound which is a mammalian NPFF receptor agonist effective to treat the abnormality. In separate embodiments, the abnormality is a regulation of a steroid hormone disorder, an epinephrine release disorder, a gastrointestinal disorder, a cardiovascular disorder, an electrolyte balance disorder, hypertension, diabetes, a respiratory disorder, asthma, a reproductive function disorder, an immune disorder, an endocrine disorder, a musculoskeletal disorder, a neuroendocrine disorder, a

cognitive disorder, a memory disorder, a sensory modulation and transmission disorder, a motor coordination disorder, a sensory integration disorder, a motor integration disorder, a dopaminergic function disorder, an appetite disorder, obesity, a sensory transmission disorder, an olfaction disorder, a sympathetic innervation disorder, an affective disorder, pain, psychotic behavior, morphine tolerance, opiate addiction or migraine.

This invention provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by decreasing the activity of a mammalian NPFF receptor which comprises administering to the subject an amount of a compound which is a mammalian NPFF receptor antagonist effective to treat the abnormality. In separate embodiments, the abnormality is a regulation of a steroid hormone disorder, an epinephrine release disorder, a gastrointestinal disorder, a cardiovascular disorder, an electrolyte balance disorder, hypertension, diabetes, a respiratory disorder, asthma, a reproductive function disorder, an immune disorder, an endocrine disorder, a musculoskeletal disorder, a neuroendocrine disorder, a cognitive disorder, a memory disorder, a sensory modulation and transmission disorder, a motor coordination disorder, a sensory integration disorder, a motor integration disorder, a dopaminergic function disorder, an appetite disorder, obesity, a sensory transmission disorder, an olfaction disorder, a sympathetic innervation disorder, an affective disorder, pain psychotic behavior, morphine tolerance, opiate addiction or migraine.

This invention also provides the use of mammalian NPFF receptors for analgesia.

This invention provides a process for making a composition of matter which specifically binds to a mammalian NPFF receptor which comprises identifying a chemical compound using any of the processes described herein for identifying a compound which binds to and/or activates or inhibits activation of a mammalian NPFF receptor and then synthesizing the chemical compound or a novel structural and functional analog or homolog thereof. In one embodiment, the mammalian NPFF receptor is a human NPFF1 receptor. In another embodiment, the mammalian NPFF receptor is a human NPFF2 receptor.

This invention further provides a process for preparing a pharmaceutical composition which comprises admixing a pharmaceutically acceptable carrier and a pharmaceutically acceptable amount of a chemical compound identified by any of the processes described herein for identifying a compound which binds to and/or activates or inhibits activation of a mammalian NPFF receptor or a novel structural and functional analog or homolog thereof. In one embodiment, the mammalian NPFF receptor is a human NPFF1 receptor. In another embodiment, the mammalian NPFF receptor is a human NPFF2 receptor.

Thus, once the gene for a targeted receptor subtype is cloned, it is placed into a recipient cell which then expresses the targeted receptor subtype on its surface. This cell, which expresses a single population of the targeted human receptor subtype, is then propagated resulting in the establishment of a cell line. This cell line, which constitutes a drug discovery system, is used in two different types of assays: binding assays and functional assays. In binding assays, the affinity of a compound for both the receptor subtype that is the target

of a particular drug discovery program and other receptor subtypes that could be associated with side effects are measured. These measurements enable one to predict the potency of a compound, as well as the degree of selectivity that the compound has for the targeted receptor subtype over other receptor subtypes. The data obtained from binding assays also enable chemists to design compounds toward or away from one or more of the relevant subtypes, as appropriate, for optimal therapeutic efficacy. In functional assays, the nature of the response of the receptor subtype to the compound is determined. Data from the functional assays show whether the compound is acting to inhibit or enhance the activity of the receptor subtype, thus enabling pharmacologists to evaluate compounds rapidly at their ultimate human receptor subtypes targets permitting chemists to rationally design drugs that will be more effective and have fewer or substantially less severe side effects than existing drugs.

Approaches to designing and synthesizing receptor subtype-selective compounds are well known and include traditional medicinal chemistry and the newer technology of combinatorial chemistry, both of which are supported by computer-assisted molecular modeling. With such approaches, chemists and pharmacologists use their knowledge of the structures of the targeted receptor subtype and compounds determined to bind and/or activate or inhibit activation of the receptor subtype to design and synthesize structures that will have activity at these receptor subtypes.

Combinatorial chemistry involves automated synthesis of a variety of novel compounds by assembling them using

different combinations of chemical building blocks. The use of combinatorial chemistry greatly accelerates the process of generating compounds. The resulting arrays of compounds are called libraries and are used to screen for compounds ("lead compounds") that demonstrate a sufficient level of activity at receptors of interest. Using combinatorial chemistry it is possible to synthesize "focused" libraries of compounds anticipated to be highly biased toward the receptor target of interest.

Once lead compounds are identified, whether through the use of combinatorial chemistry or traditional medicinal chemistry or otherwise, a variety of homologs and analogs are prepared to facilitate an understanding of the relationship between chemical structure and biological or functional activity. These studies define structure activity relationships which are then used to design drugs with improved potency, selectivity and pharmacokinetic properties. Combinatorial chemistry is also used to rapidly generate a variety of structures for lead optimization. Traditional medicinal chemistry, which involves the synthesis of compounds one at a time, is also used for further refinement and to generate compounds not accessible by automated techniques. Once such drugs are defined the production is scaled up using standard chemical manufacturing methodologies utilized throughout the pharmaceutical and chemistry industry.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

EXPERIMENTAL DETAILS

Materials and methods

Cloning of rat and human NPFF1 receptor

MOPAC (Mixed Oligonucleotide Primed Amplification of cDNA

100ng of rat genomic DNA (Clonetech, Palo Alto, CA) was used for degenerate MOPAC PCR using Taq DNA polymerase (Boehringer-Mannheim, Indianapolis, IN) and the following degenerate oligonucleotides: JAB126, designed based on an alignment of the sixth transmembrane domain of more than 180 members of the rhodopsin superfamily of G protein-coupled receptors; and JAB108, designed based on an alignment of the seventh transmembrane domain of the same rhodopsin superfamily.

The conditions for the MOPAC PCR reaction were as follows: 3 minute hold at 94°C; 10 cycles of 1 minute at 94°C, 1 minute 45 seconds at 44°C, 2 minutes at 72°C; 30 cycles of 94°C for 1 minute, 49°C for 1 minute 45 seconds, 2 minutes at 72°C; 4 minute hold at 72°C; 4°C until ready for agarose gel electrophoresis.

The products were run on a 1% agarose TAE gel and bands of the expected size (~150bp) were cut from the gel, purified using the QIAQUICK gel extraction kit (QIAGEN, Chatsworth, CA), and subcloned into the TA cloning vector (Invitrogen, San Diego, CA). White (insert-containing) colonies were picked and subjected to PCR using pCR2.1 vector primers JAB1 and JAB2 using the Expand Long Template PCR System and the following protocol: 94°C hold for 3 minutes; 35 cycles of 94°C for 1 minute, 68°C for 1 minute 15 seconds; 2 minute hold at 68°C, 4°C hold until products were ready for purification. PCR products were purified by

isopropanol precipitation (10 μl PCR product, 18 μl low TE, 10.5 μl 2M $NaClO_4$ and 21.5 μl isopropanol) and sequenced using the ABI Big Dye cycle sequencing protocol and ABI 377 sequencers (ABI, Foster City, CA). Nucleotide and amino acid sequence analyses were performed using the Wisconsin Package (GCG, Genetics Computer Group, Madison, WI). Two PCR products produced from rat genomic cDNA (MPR3-RGEN-31 and MPR3-RGEN-45) were determined to be identical clones of a novel G protein-coupled receptor-like sequence based on database searches and its homology to other known G protein-coupled receptors (~30-40% amino acid identity to dopamine D2, orexin, galanin, angiotensin 1 and $5\text{-}HT_{2b}$ receptors). This novel sequence was designated SNORF2.

Cloning of the full-length coding sequence of SNORF2 (rat NPFF1)

Pools of the rat hypothalamic cDNA library "I" were screened by PCR with SNORF2-specific primers JAB208 and JAB209 and the Expand Long Template PCR system (Boehringer-Mannheim, Indianapolis, IN) with the following PCR protocol: 94°C hold for 3 minutes; 40 cycles of 94°C for 1 minute, 68°C for 2 minutes; 4 minute hold at 68°C; 4°C hold until the samples are run on a gel. This screen yielded a positive pool I36E and a positive sub-pool I36E-17. High stringency hybridization of isolated colonies from I36E-17 with the SNORF2-specific oligonucleotide probe JAB211 and subsequent PCR testing of positive colonies indicated that the isolated clone I36E-17-1B-1 contained at least a partial clone of SNORF2. Sequencing of I36E-17-1B-1 revealed that this insert contained the coding region from the TMIII-TMIV loop through the stop codon, including some 3' untranslated sequence. From this sequence, a new forward primer,

JAB221, was designed in TMV. PCR screening of a second rat hypothalamic cDNA library "J" with primers JAB221 and JAB209, and subsequent colony hybridization with the JAB211 probe on a low complexity positive sub-pool resulted in the isolation of a SNORF2 clone J-13-16-A1. Full-length double-stranded sequence of SNORF2 was determined by sequencing both strands of the J-13-16-A1 plasmid using an ABI 377 sequencer as described above. This insert is about 2.8 kb in length with an approximately 200 bp 5' untranslated region, a 1296 bp coding region, and a 1.3 kb 3'untranslated region. The clone is also in the correct orientation for expression in the mammalian expression vector pEXJ.T7. This construct of SNORF2 in pEXJ.T7 was designated BN-6. The full length SNORF2 was determined to be most like the orexin 1 receptor (45% DNA identity, 35% amino acid identity), orexin 2 receptor (40% DNA identity, 32% amino acid identity), and NPY2 receptor (47% DNA identity, 29% amino acid identity), although several other G protein-coupled receptors also displayed significant homology. There were no sequences in the Genbank databases (genembl, sts, est, gss, or swissprot) that were identical to SNORF2. SNORF2 also showed significant homology (85% nucleotide identity, 93% amino acid identity) to a partial G protein-coupled receptor fragment in the Synaptic Pharmaceutical Corporation in-house database, designated PLC29b. PLC29b, which includes part of the amino terminus through TMIII, was originally isolated from a human genomic library using oligonucleotide probes for NPY4. Subsequent screening of a human hippocampal cDNA library yielded an overlapping sequence extending into TMIV. Based on sequence similarity, this human sequence appears to be a partial clone of the human homolog of SNORF2.

The following is a list of primers and their associated sequences which were used in the cloning of these receptors:

JAB126: 5'-GYNTWYRYNNTNWSNTGGHTNCC-3' (Seq. ID No. 9)
JAB108: 5'-AVNADNGBRWAVANNANNGGRTT-3' (Seq. ID No. 10)
JAB1: 5'-TTATGCTTCCGGCTCGTATGTTGTG-3' (Seq. ID No. 11)
JAB2: 5'-ATGTGCTGCAAGGCGATTAAGTTGGG-3' (Seq. ID No. 12)
JAB208: 5'-GGTGCTGCTGCTGCTCATCGACTATG-3' (Seq. ID No. 13)
JAB209: 5'-TTGGCGCTGCTGTGGAAGAAGGCCAG-3' (Seq. ID No. 14)
JAB221: 5'-CGGTGCTCTTCGCGCACATCTACC-3' (Seq. ID No. 15)
JAB211: 5'-TGCCAAGGGGAAGGCGTAGACCGACAGCAGGTGCAGTTGCA GCTCGATCAGCTCCCCATA-3' (Seq. ID No. 16)

Isolation of the full-length human SNORF2 receptor gene (human NPFF1)

The full-length, intronless version of the human NPFF1 receptor gene may be isolated using standard molecular biology techniques and approaches such as those briefly described below:

Approach #1: To obtain a full-length human NPFF1 receptor, a human cosmid library was screened with a ^{32}P-labeled oligonucleotide probe, BB609, corresponding to the 2/3 loop of the PLC29b clone. A positive clone was isolated and partially sequenced, revealing part of the amino terminus and TMs I and II.

The full-length sequence may be obtained by sequencing this cosmid clone with additional sequencing primers. Since at least two introns are present in this gene, one in the amino terminus and one just after the third transmembrane domain, the full-length intronless gene may

be obtained from cDNA using standard molecular biology techniques. For example, a forward PCR primer designed in the 5'UT and a reverse PCR primer designed in the 3'UT may be used to amplify a full-length, intronless gene from cDNA. RT-PCR localization has identified several human tissues which could be used for this purpose, including cerebellum, spinal cord, hippocampus, lung and kidney. Standard molecular biology techniques could be used to subclone this gene into a mammalian expression vector.

Approach #2: Standard molecular biology techniques could be used to screen commercial human cDNA phage libraries by hybridization under high stringency with a ^{32}P-labeled oligonucleotide probe, BB609, corresponding to the 2/3 loop of the PLC29b clone. One may isolate a full-length human NPFF1 by obtaining a plaque purified clone from the lambda libraries and then subjecting the clone to direct DNA sequencing using primers from the PLC29b sequence. Alternatively, standard molecular biology techniques could be used to screen in-house human cDNA plasmid libraries by PCR amplification of library pools using primers to the human NPFF1 sequence (BB629, forward primer in TMI, and A71, reverse primer in TMIV). A full-length clone could be isolated by Southern hybridization of colony lifts of positive pools with a ^{32}P-labeled oligonucleotide probe, BB609, corresponding to the 2/3 loop of the PLC29b clone.

Approach #3: As yet another alternative method, one could utilize 3' and 5' RACE to generate PCR products from human cDNA expressing human NPFF1 (for example, cerebellum, spinal cord, hippocampus, lung and kidney), which contain the additional sequences of human NPFF1. For 5' RACE, a reverse primer derived from PLC29b between the amino terminus and TM IV could be used to amplify the additional

amino terminus sequence for hNPFF1. For 3' RACE, a forward primer derived from PLC29b between the amino terminus and TM IV could be used to amplify the additional 3' sequence for hNPFF1, including TMs 5-7 and the COOH terminus. These RACE PCR product could then be sequenced to determine the missing sequence. This new sequence could then be used to design a forward PCR primer in the 5'UT and a reverse primer in the 3'UT. These primers could then be used to amplify a full-length hNPFF1 clone from human cDNA sources known to express NPFF1 (for example, cerebellum, spinal cord, hippocampus, lung and kidney).

BB609: 5'-CCACCCTTGTGGACAACCTCATCACTGGGTGGCCCTTCGACAATGCC ACATGC-3' (Seq. ID No. 17)

BB629: 5'-CTGCTCTGCATGGTGGGCAACACC-3' (Seq. ID No. 18)

A71: 5'-GACGGCGATGGTGACGAGCGC-3' (Seq. ID No. 19)

Cloning of human NPFF1 receptor

The sequence of the human NPFF1 (hNPFF1) receptor from the initiating methionine to TMIV was determined to be present in a partial clone, plc29b, found in a Synaptic Pharmaceutical Corporation in-house database. In order to isolate the full-length hNPFF1 receptor cDNA, a human cosmid library (Stratagene) was screened with a ^{32}P-labeled probe (BB609) corresponding to the II/III loop of plc29b. Partial DNA sequencing of one positive clone from this library, COS28a revealed similar sequence as had been previously shown for plc29b, with an intron downstream of TMIII. In order to obtain sequence in the 3' end of hNPFF1, COS28a was amplified with a vector primer and

BB702, BB703 or BB704, forward primers in TMIV. DNA sequencing of these PCR products resulted in the identification of TMIV through the stop codon.

Next, an in-house human spinal cord library was screened by PCR using a forward primer in the region of the initiating methionine (BB729) and a reverse primer corresponding to TMIV (BB728). One positive pool, W4, was subdivided and a positive sub-pool was screened by colony hybridization with a ^{32}P-labeled probe from TMII, BB676. Plasmid DNA was isolated for clone W4-18-4, renamed BO98, and DNA sequencing revealed that it was full-length but in the wrong orientation for expression in the expression vector pEXJ. To obtain a full-length hNPFF1 construct in the correct orientation, BO98 was amplified with BB757, a forward primer at the initiating methionine which contained an upstream *Bam*HI site, and BB758, a reverse primer at the stop codon which contained a *Eco*RI site. The products from 3 independent PCR reactions were ligated into pcDNA3.1+ and transformed into DH5α cells. The sequence of one of these transformants, 3.3, was identical to the hNPFF1 sequence previously determined from the consensus of BO98, COS28a and plc29b. Clone 3.3 was renamed BO102.

The hNPFF1 clone contains an open reading frame with 1293 nucleotides and predicts a protein of 430 amino acids (Figures 11 and 12). Hydrophobicity analysis reveals seven hydrophobic domains which are presumed to be transmembrane domains (Figure 13). The sequence of hNPFF1 was determined to be most similar to the rat NPFF1 (86% nucleotide identity, 87% amino acid identity) and human NPFF2 (56% nucleotide identity, 49% amino acid identity (Figure 14)). The human NPFF1 receptor also shares

homology with human orexin$_1$ (53% nucleotide identity, 35% amino acid identity), human orexin$_2$ (43% nucleotide identity, 33% amino acid identity), human NPY$_2$ (47% nucleotide identity, 31% amino acid identity), human CCK$_A$ (46% nucleotide identity, 32% amino acid identity), and human CCK$_B$ (46% nucleotide identity, 26% amino acid identity).

The following primers and probes were used in the cloning of hNPFF1:

BB676: 5'-GTCACCAACATGTTCATCCTCAACCTGGCTGTCAGTGACCTGCT GGTGGGCATCTTCTGCATGCC-3' (Seq. ID No. 20)

BB702: 5'-GCGAGAAGCTGACCCTGCGGAAGG-3' (Seq. ID No. 21)

BB703: 5'-TCGTCACCATCGCCGTCATCTGGG-3' (Seq. ID No. 22)

BB704: 5'-CGTCATCTGGGCCGAGGGACACAG-3' (Seq. ID No. 23)

BB728: 5'-TGACGGCGATGGTGACGAGCGCC-3' (Seq. ID No. 24)

BB729: 5'-CAGCCTCCCAACAGCAGTTGGCC-3' (Seq. ID No. 25)

BB757: 5'-TAGCAAGGATCCGCATATGGAGGGGGAGCCCTCCC-3' (Seq. ID No. 26)

BB758: 5'-CTTCATGAATTCATCGCCTGCATGTATCTCGTGTCC-3' (Seq. ID No. 27)

Cloning of human NPFF2 receptor

Discovery of an expressed sequence tag (EST) AA449919 in GENEMBL homologous to rNPFF1 (hNPFF2)

A FASTA search of GENEMBL with the full-length sequence of rat NPFF1 (rNPFF1) resulted in the identification of an EST (Accession number AA449919) with a high degree of homology to NPFF1 (57% identity at the DNA level). AA449919 is a 532 bp sequence annotated in Genbank as "Soares total fetus Nb2HF8 9w Homo sapiens cDNA clone 788698 5' similar to SW:NYR_DROME P25931 NEUROPEPTIDE Y RECEPTOR," which when translated corresponds to the region

between the first extracellular loop and the beginning of the sixth transmembrane domain of rNPFF1. GAP analysis of AA449919 with rNPFF1 indicated that there is 57% DNA identity and a 50% amino acid identity between the two receptor sequences over this region. AA449919 displays 60% DNA identity and 59% amino acid identity over the region that overlaps with the known sequence for hNPFF1 (first extracellular loop to TM4), while over the same range rNPFF1 is 62% and 61% identical to AA449919 at the DNA and amino acid levels, respectively. In comparison, hNPFF1 and rNPFF1 share 86% DNA identity and 92 % amino acid identity over this region. Given the strong degree of identity between AA449919 and rNPFF1, AA449919 was given the name NPFF-like (hNPFF2).

Cloning the full-length sequence of (NPFF-like) hNPFF2

To determine the full-length coding sequence of AA449919, 5'/3' Rapid Amplification of cDNA ends (RACE) was performed on Clontech Human Spleen Marathon-Ready cDNA (Clontech, Palo Alto, CA). For 5' RACE, 5μl template (human spleen Marathon-Ready cDNA was amplified with oligonucleotide primers JAB256 and AP1, the Expand Long DNA Template PCR System (Boehringer-Mannheim, Indianapolis, IN) and the following PCR protocol were used: 94°C hold for 3 minutes; 5 cycles of 94°C for 30 seconds, 72°C for 4 minutes; 5 cycles of 94°C for 30 seconds, 70°C for 4 minutes; 30 cycles of 94°C for 30 seconds, 68°C for 4 minutes; 68°C hold for 4 minutes; 4°C hold until products were ready to be loaded on a gel. 1μl of this reaction was subjected to a second round of amplification with primers JAB260 and AP2 and the same PCR protocol. For 3' RACE, 5 μl human spleen Marathon-Ready cDNA was subjected to PCR with primers JAB257 and AP1 with the same PCR protocol that was used for 5' RACE. 1μl of

this reaction was subjected to another round of amplification using AP2 and JAB258 and the same PCR conditions.

The products were run on a 1% agarose TAE gel and bands greater than 500 bp were extracted from the gel using the QIAQUICK gel extraction kit (QIAGEN, Chatsworth, CA). 5 μl of each purified band from the 5' and 3' RACE reactions were directly sequenced with primers JAB261 (5' products) and JAB259 (3' products) using the ABI Big Dye cycle sequencing protocol and ABI377 sequencers (ABI, Foster City, CA). The Wisconsin Package (GCG, Genetics Computer Group, Madison, WI) and Sequencer 3.0 (Gene Codes Corporation, Ann Arbor, MI) were used to put together the full-length contiguous sequence of hNPFF2 from the AA449919 EST and the RACE products.

To attain the full-length hNPFF-like (hNPFF2) coding sequence for expression, human spinal cord cDNA was amplified in eight independent PCR reactions using the Expand Long Template PCR System with buffer I (four of the eight reactions) or buffer 3 (4 reactions) and two oligonucleotide primers with restriction sites incorporated into their 5' ends: BB675 is a forward primer upstream of the initiating methionine and contains a BamHI site, and BB663. The PCR conditions for this reaction were as follows: 94°C hold for 5 minutes; 37 cycles of 94°C for 30 seconds, 64°C for 30 seconds, 68°C for 2 minutes; a 7 minute hold at 68°C, and a 4°C hold until products were ready to be loaded on a gel. The products were electrophoresed on a 1% agarose TAE gel, and a band of approximately 1.35 kb was cut and purified using the QIAQUICK gel extraction kit. The purified bands of seven of the eight reactions were cut with BamHI and EcoRI, gel

purified again using the same method, and ligated into pcDNA3.1(+) (Invitrogen, Carlsbad, CA). Eighteen colonies from the subsequent transformations were picked and determined to be positive for NPFF-like by PCR. Eight of these 18 clones were fully sequenced, and one of these, BO89, was determined to be a full length clone with no point mutations. This construct was renamed pcDNA3.1-hNPFF2b.

For expression of NPFF-like in oocytes, one ul of each of these eight ligations of the BB675-BB663 PCR product into pcDNA3.1(+) was subjected to PCR with AN35, a pcDNA3.1 primer at the CMV promoter site, and the 3' NPFF-like primer BB663 using the Expand Long Template PCR System and the following PCR protocol: 94°C hold for 3 minutes; 37 cycles of 94°C for 30 seconds, 65°C for 30 seconds, 68°C for 2 minutes; a 7 minute hold at 68°C, and a 4°C hold until products were ready for in vitro transcription. Of the seven PCR reactions, six yielded products of the expected size.

The following is a list of primers and their associated sequences which were used in the cloning of this receptor (hNPFF2):

AN35: 5'-CGTGTACGGTGGGAGGTCTATATAAGCAGAG-3' (Seq. ID No. 28)

AP1: 5'-CCATCCTAATACGACTCACTATAGGGC-3' (Seq. ID No. 29)

AP2: 5'-ACTCACTATAGGGCTCGAGCGGC-3' (Seq. ID No. 30)

JAB256: 5'-TGATAGTGAGCTTTGGTTTAAAAGGG-3' (Seq. ID No. 31)

JAB257: 5'-GAAGATCTACACCACTGTGCTGTTTG-3' (Seq. ID No. 32)

JAB258: 5'-AACATCTACCTGGCTCCCCTCTCCC-3' (Seq. ID No. 33)

JAB259: 5'-TTGTCATCATGTATGGAAGGATTGG-3' (Seq. ID No. 34)

JAB260: 5'-GACCACACACTGGAACCTATCTAC-3' (Seq. ID No. 35)

JAB261: 5'-GCAATTGCAACTAACGTAAAGACTG-3' (Seq. ID No. 36)

BB675: 5'-TAGCAAGGATCCGAGGTTCATCATGAATGAGAAATGG-3' (Seq. ID No. 37)

BB663: 5'-CTTCATGAATTCGCGTAGTAGAGTTAGGATTATCAC-3' (Seq. ID No. 38)

For expression of NPFF2, mRNA transcripts were generated as described for NPFF1, using PCR products from ligation reactions or linearized DNA from BO89 as DNA templates. Oocytes were injected with 5-50 ng NPFF2 mRNA and incubated as previously described.

Cell culture

COS-7 cells are grown on 150 mm plates in DMEM with supplements (Dulbecco's Modified Eagle Medium with 10% bovine calf serum, 4 mM glutamine, 100 units/ml penicillin/100 μg/ml streptomycin) at 37°C, 5% CO_2. Stock plates of COS-7 cells are trypsinized and split 1:6 every 3-4 days.

Human embryonic kidney 293 cells are grown on 150 mm plates in DMEM with supplements (10% bovine calf serum, 4 mM glutamine, 100 units/ml penicillin/100 μg/ml streptomycin) at 37°C, 5% CO_2. Stock plates of 293 cells are trypsinized and split 1:6 every 3-4 days.

Mouse fibroblast LM(tk-) cells are grown on 150 mm plates in D-MEM with supplements (Dulbecco's Modified Eagle Medium with 10% bovine calf serum, 4 mM glutamine, 100 units/ml penicillin/100 μg/ml streptomycin) at 37°C, 5% CO_2. Stock plates of LM(tk-) cells are trypsinized and split 1:10 every 3-4 days.

Chinese hamster ovary (CHO) cells were grown on 150 mm plates in HAM's F-12 medium with supplements (10% bovine

calf serum, 4 mM L-glutamine and 100 units/ml penicillin/ 100 ug/ml streptomycin) at 37°C, 5% CO_2. Stock plates of CHO cells are trypsinized and split 1:8 every 3-4 days.

Mouse embryonic fibroblast NIH-3T3 cells are grown on 150 mm plates in Dulbecco's Modified Eagle Medium (DMEM) with supplements (10% bovine calf serum, 4 mM glutamine, 100 units/ml penicillin/100 μg/ml streptomycin) at 37°C, 5% CO2. Stock plates of NIH-3T3 cells are trypsinized and split 1:15 every 3-4 days.

Sf9 and Sf21 cells are grown in monolayers on 150 mm tissue culture dishes in TMN-FH media supplemented with 10% fetal calf serum, at 27°C, no CO_2. High Five insect cells are grown on 150 mm tissue culture dishes in Ex-Cell 400™ medium supplemented with L-Glutamine, also at 27°C, no CO_2.

Transient transfection

Receptors studied may be transiently transfected into COS-7 cells by the DEAE-dextran method using 1 μg of DNA /10^6 cells (Cullen, 1987). In addition, Schneider 2 Drosophila cells may be cotransfected with vectors containing the receptor gene under control of a promoter which is active in insect cells, and a selectable resistance gene, eg., the G418 resistant neomycin gene, for expression of the polypeptides disclosed herein.

Stable transfection

DNA encoding the human receptor disclosed herein may be co-transfected with a G-418 resistant gene into the human embryonic kidney 293 cell line by a calcium phosphate transfection method (Cullen, 1987). Stably transfected cells are selected with G-418.

Membrane preparations

LM(tk-) cells stably transfected with the DNA encoding the human receptor disclosed herein may be routinely converted from an adherent monolayer to a viable suspension. Adherent cells are harvested with trypsin at the point of confluence, resuspended in a minimal volume of complete DMEM for a cell count, and further diluted to a concentration of 10^6 cells/ml in suspension media (10% bovine calf serum, 10% 10X Medium 199 (Gibco), 9 mM $NaHCO_3$, 25 mM glucose, 2 mM L-glutamine, 100 units/ml penicillin/100 μg/ml streptomycin, and 0.05% methyl cellulose). Cell suspensions are maintained in a shaking incubator at 37°C, 5% CO_2 for 24 hours. Membranes harvested from cells grown in this manner may be stored as large, uniform batches in liquid nitrogen. Alternatively, cells may be returned to adherent cell culture in complete DMEM by distribution into 96-well microtiter plates coated with poly-D-lysine (0.01 mg/ml) followed by incubation at 37°C, 5% CO_2 for 24 hours.

Generation of baculovirus

The coding region of DNA encoding the human receptors disclosed herein may be subcloned into pBlueBacIII into existing restriction sites or sites engineered into sequences 5′ and 3′ to the coding region of the polypeptides. To generate baculovirus, 0.5 μg of viral DNA (BaculoGold) and 3 μg of DNA construct encoding a polypeptide may be co-transfected into 2 x 10^6 *Spodoptera frugiperda* insect Sf9 cells by the calcium phosphate co-precipitation method, as outlined by Pharmingen (in "Baculovirus Expression Vector System: Procedures and Methods Manual"). The cells then are incubated for 5 days at 27°C.

The supernatant of the co-transfection plate may be collected by centrifugation and the recombinant virus plaque purified. The procedure to infect cells with virus, to prepare stocks of virus and to titer the virus stocks are as described in Pharmingen's manual.

Radioligand binding assays

Cells may be screened for the presence of endogenous human receptor using radioligand binding or functional assays (described in detail in the following experimental description). Cells with either no or a low level of the endogenous human receptors disclosed herein present may be transfected with the human receptors.

Transfected cells from culture flasks are scraped into 5 ml of 20 mM Tris-HCl, 5mM EDTA, pH 7.5, and lysed by sonication. The cell lysates are centrifuged at 1000 rpm for 5 min. at 4°C, and the supernatant is centrifuged at 30,000 x g for 20 min. at 4°C. The pellet is suspended in binding buffer (50 mM Tris-HCl, 60 mM NaCl, 1 mM MgCl, 33μM EDTA, 33 μM EGTA at pH 7.4 supplemented with 0.2% BSA, 2 μg/ml aprotinin, and 20 μM bestatin). Optimal membrane suspension dilutions, defined as the protein concentration required to bind less than 10% of the added radioligand, are added to 96-well polpropylene microtiter plates containing ^{3}H-labeled compound, unlabeled compounds, and binding buffer to a final volume of 250 μl. In equilibrium saturation binding assays membrane preparations are incubated in the presence of increasing concentrations of [^{3}H]-labeled compound. The binding affinities of the different compounds are determined in equilibrium competition binding assays, using [^{125}I]-labeled compound in the presence of ten to twelve different concentrations of the displacing ligands.

Competition assay: 50pM radioligand, 10 - 12 points. Binding reaction mixtures are incubated for 2 hr at 25°C, and the reaction stopped by filtration through a double layer of GF filters treated with 0.1% polyethyleneimine, using a cell harvester. Wash buffer: 50mM Tris-HCl, 0.1% BSA. Radioactivity may be measured by scintillation counting and data are analyzed by a computerized non-linear regression program. Non-specific binding is defined as the amount of radioactivity remaining after incubation of membrane protein in the presence of 1μM final concentration unlabeled. Protein concentration may be measured by the Bradford method using Bio-Rad Reagent, with bovine serum albumin as a standard.

Functional assays

Cells may be screened for the presence of endogenous mammalian receptor using radioligand binding or functional assays (described in detail in the above or following experimental description, respectively). Cells with no or a low level of endogenous receptor present may be transfected with the mammalian receptor for use in the following functional assays.

A wide spectrum of assays can be employed to screen for the presence of receptor ligands. These range from traditional measurements of phosphatidyl inositol, cAMP, Ca^{++}, and K^{+}, for example; to systems measuring these same second messengers but which have been modified or adapted to be higher throughput, more generic, and more sensitive; to cell based platforms reporting more general cellular events resulting from receptor activation such as metabolic changes, differentiation, and cell division/proliferation, for example; to high level organism assays which monitor complex physiological or

behavioral changes thought to be involved with receptor activation including cardiovascular, analgesic, orexigenic, anxiolytic, and sedation effects, for example.

Cyclic AMP (cAMP) formation assay

The receptor-mediated inhibition of cyclic AMP (cAMP) formation may be assayed in transfected cells expressing the mammalian receptors. Cells are plated in 96-well plates and incubated in Dulbecco's phosphate buffered saline (PBS) supplemented with 10 mM HEPES, 5mM theophylline, 2 μg/ml aprotinin, 0.5 mg/ml leupeptin, and 10 μg/ml phosphoramidon for 20 min at 37°C, in 5% CO_2. Test compounds are added and incubated for an additional 10 min at 37°C. The medium is then aspirated and the reaction stopped by the addition of 100 mM HCl. The plates are stored at 4°C for 15 min, and the cAMP content in the stopping solution measured by radioimmunoassay. Radioactivity may be quantified using a gamma counter equipped with data reduction software.

Arachidonic acid release assay

Cells stably transfected with the mammalian receptor are seeded into 96 well plates and grown for 3 days in HAM's F-12 with supplements. ^{3}H-arachidonic acid (specific activity = 0.75 μCi/ml) is delivered as a 100 μL aliquot to each well and samples were incubated at 37° C, 5% CO_2 for 18 hours. The labeled cells are washed three times with 200 μL HAM's F-12. The wells are then filled with medium (200 μL) and the assay is initiated with the addition of peptides or buffer (22 μL). Cells are incubated for 30 min at 37°C, 5% CO_2. Supernatants are transferred to a microtiter plate and evaporated to dryness at 75°C in a vacuum oven. Samples are then dissolved and resuspended in 25 μL distilled water.

Scintillant (300 μL) is added to each well and samples are counted for ^3H in a Trilux plate reader. Data are analyzed using nonlinear regression and statistical techniques available in the GraphPAD Prism package (San Diego, CA).

Intracellular calcium mobilization assay

The intracellular free calcium concentration may be measured by microspectroflourometry using the fluorescent indicator dye Fura-2/AM (Bush et al, 1991). Stably transfected cells are seeded onto a 35 mm culture dish containing a glass coverslip insert. Cells are washed with HBS and loaded with 100 μL of Fura-2/AM (10 μM) for 20 to 40 min. After washing with HBS to remove the Fura-2/AM solution, cells are equilibrated in HBS for 10 to 20 min. Cells are then visualized under the 40X objective of a Leitz Fluovert FS microscope and fluorescence emission is determined at 510 nM with excitation wavelengths alternating between 340 nM and 380 nM. Raw fluorescence data are converted to calcium concentrations using standard calcium concentration curves and software analysis techniques.

Phosphoinositide metabolism assay

Cells stably expressing the mammalian receptor cDNA are plated in 96-well plates and grown to confluence. The day before the assay the growth medium is changed to 100 μl of medium containing 1% serum and 0.5 μCi [^3H]myo-inositol, and the plates are incubated overnight in a CO_2 incubator (5% CO_2 at 37^{0}C). Alternatively, arachidonic acid release may be measured if [^3H]arachidonic acid is substituted for the [^3H]myo-inositol. Immediately before the assay, the medium is removed and replaced by 200 μL of PBS containing 10 mM LiCl, and the cells are equilibrated with the new medium for 20 min. During this interval cells are also

equilibrated with the antagonist, added as a 10 μL aliquot of a 20-fold concentrated solution in PBS. The [^{3}H] inositol-phosphates accumulation from inositol phospholipid metabolism may be started by adding 10 μL of a solution containing the agonist. To the first well 10 μL may be added to measure basal accumulation, and 11 different concentrations of agonist are assayed in the following 11 wells of each plate row. All assays are performed in duplicate by repeating the same additions in two consecutive plate rows. The plates are incubated in a CO_2 incubator for 1 hr. The reaction may be terminated by adding 15 μL of 50% v/v trichloroacetic acid (TCA), followed by a 40 min. incubation at 4 ^{0}C. After neutralizing TCA with 40 μL of 1 M Tris, the content of the wells may be transferred to a Multiscreen HV filter plate (Millipore) containing Dowex AG1-X8 (200-400 mesh, formate form). The filter plates are prepared adding 200 μL of Dowex AG1-X8 suspension (50% v/v, water: resin) to each well. The filter plates are placed on a vacuum manifold to wash or elute the resin bed. Each well is washed 2 times with 200 μL of water, followed by 2 x 200 μL of 5 mM sodium tetraborate/60 mM ammonium formate. The [^{3}H] IPs are eluted into empty 96-well plates with 200 μL of 1.2 M ammonium formate/0.1 formic acid. The content of the wells is added to 3 ml of scintillation cocktail, and the radioactivity is determined by liquid scintillation counting.

GTPγS functional assay

Membranes from cells transfected with the mammalian receptors are suspended in assay buffer (50 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.4) supplemented with 0.1% BSA, 0.1% bacitracin and 10 μM GDP. Membranes are incubated on ice for 20 minutes, transferred to a 96-well Millipore

microtiter GF/C filter plate and mixed with GTPγ^{35}S (e.g., 250,000 cpm/sample, specific activity ~1000 Ci/mmol) plus or minus GTPγS (final concentration = 100 μM). Final membrane protein concentration ≈ 90 μg/ml. Samples are incubated in the presence or absence of porcine galanin (final concentration = 1 μM) for 30 min. at room temperature, then filtered on a Millipore vacuum manifold and washed three times with cold assay buffer. Samples collected in the filter plate are treated with scintillant and counted for ^{35}S in a Trilux (Wallac) liquid scintillation counter. It is expected that optimal results are obtained when the mammalian receptor membrane preparation is derived from an appropriately engineered heterologous expression system, i.e., an expression system resulting in high levels of expression of the mammalian receptor and/or expressing G-proteins having high turnover rates (for the exchange of GDP for GTP). GTPγS assays are well-known in the art, and it is expected that variations on the method described above, such as are described by e.g., Tian et al. (1994) or Lazareno and Birdsall (1993), may be used by one of ordinary skill in the art.

MAP kinase assay

MAP kinase (mitogen activated kinase) may be monitored to evaluate receptor activation. MAP kinase is activated by multiple pathways in the cell. A primary mode of activation involves the ras/raf/MEK/MAP kinase pathway. Growth factor (tyrosine kinase) receptors feed into this pathway via SHC/Grb-2/SOS/ras. Gi coupled receptors are also known to activate ras and subsequently produce an activation of MAP kinase. Receptors that activate phospholipase C (Gq and G11) produce diacylglycerol (DAG) as a consequence of phosphatidyl inositol hydrolysis. DAG activates protein kinase C which in turn phosphorylates

MAP kinase.

MAP kinase activation can be detected by several approaches. One approach is based on an evaluation of the phosphorylation state, either unphosphorylated (inactive) or phosphorylated (active). The phosphorylated protein has a slower mobility in SDS-PAGE and can therefore be compared with the unstimulated protein using Western blotting. Alternatively, antibodies specific for the phosphorylated protein are available (New England Biolabs) which can be used to detect an increase in the phosphorylated kinase. In either method, cells are stimulated with the mitogen and then extracted with Laemmli buffer. The soluble fraction is applied to an SDS-PAGE gel and proteins are transferred electrophoretically to nitrocellulose or Immobilon. Immunoreactive bands are detected by standard Western blotting technique. Visible or chemiluminescent signals are recorded on film and may be quantified by densitometry.

Another approach is based on evaluation of the MAP kinase activity via a phosphorylation assay. Cells are stimulated with the mitogen and a soluble extract is prepared. The extract is incubated at 30°C for 10 min with gamma-^{32}P-ATP, an ATP regenerating system, and a specific substrate for MAP kinase such as phosphorylated heat and acid stable protein regulated by insulin, or PHAS-I. The reaction is terminated by the addition of H_3PO_4 and samples are transferred to ice. An aliquot is spotted onto Whatman P81 chromatography paper, which retains the phosphorylated protein. The chromatrography paper is washed and counted for ^{32}P in a liquid scintillation counter. Alternatively, the cell extract is incubated with gamma-^{32}P-ATP, an ATP regenerating system, and

biotinylated myelin basic protein bound by streptavidin to a filter support. The myelin basic protein is a substrate for activated MAP kinase. The phosphorylation reaction is carried out for 10 min at 30°C. The extract can then by aspirated through the filter, which retains the phosphorylated myelin basic protein. The filter is washed and counted for ^{32}P by liquid scintillation counting.

Cell proliferation assay

Receptor activation of a G protein coupled receptor may lead to a mitogenic or proliferative response which can be monitored via ^{3}H-thymidine uptake. When cultured cells are incubated with ^{3}H-thymidine, the thymidine translocates into the nuclei where it is phosphorylated to thymidine triphosphate. The nucleotide triphosphate is then incorporated into the cellular DNA at a rate that is proportional to the rate of cell growth. Typically, cells are grown in culture for 1-3 days. Cells are forced into quiescence by the removal of serum for 24 hrs. A mitogenic agent is then added to the media. 24 hrs later, the cells are incubated with ^{3}H-thymidine at specific activities ranging from 1 to 10 uCi/ml for 2-6 hrs. Harvesting procedures may involve trypsinization and trapping of cells by filtration over GF/C filters with or without a prior incubation in TCA to extract soluble thymidine. The filters are processed with scintillant and counted for ^{3}H by liquid scintillation counting. Alternatively, adherant cells are fixed in MeOH or TCA, washed in water, and solubilized in 0.05% deoxycholate/0.1 N NaOH. The soluble extract is transferred to scintillation vials and counted for ^{3}H by liquid scintillation counting.

Promiscuous second messenger assays

It is possible to coax receptors of different functional classes to signal through a pre-selected pathway through the use of promiscuous G_α subunits. For example, by providing a cell based recetpor assay system with an endogenously supplied promiscuous G_α subunit such as $G_{\alpha 16}$ or a chimeric G_α subunit such as $G_{\alpha zq}$, a GPCR, which might normally prefer to couple through a specific signaling pathway (e.g., G_s, G_i, G_q, G_0, etc.), can be made to couple through the pathway defined by the promiscuous G_α subunit and upon agonist activation produce the second messenger associated with that subunit's pathway. In the case of $G_{\alpha 16}$ and/or $G_{\alpha qz}$ this would involve activation of the G_q pathway and production of the second messenger phosphotidyl inositol. Through the use of similar strategies and tools, it is possible to bias receptor signaling through pathways producing other second messengers such as Ca^{++}, cAMP, and K^+ currents, for example.

Microphysiometric measurement of receptor mediated extracellular acidification rates

Because cellular metabolism is intricately involved in a broad range of cellular events (including receptor activation of multiple messenger pathways), the use of microphysiometric measurements of cell metabolism can in principle provide a generic assay of cellular activity arising from the activation of any receptor regardless of the specifics of the receptor's signaling pathway.

General guidelines for transient receptor expression, cell preparation and microphysiometric recording are described elsewhere (Salon, J.A. and Owicki, J.A., 1996). Receptors and/or control vectors are transiently expressed in CHO-K1

cells, by liposome mediated transfection according to the manufacturers recommendations (LipofectAMINE, GibcoBRL, Gaithersburg, MD), and maintained in Ham's F-12 complete (10% serum). A total of 10μg of DNA is used to transfect each 75cm^2 flask which had been split 24 hours prior to the transfection and judged to be 70-80% confluent at the time of transfection. 24 hours post transfection, the cells are harvested and 3 x 10^5 cells seeded into microphysiometet capsules. Cells are allowed to attach to the capsule membrane for an additional 24 hours; during the last 16 hours, the cells are switched to serum-free F-12 complete to minimize ill-defined metabolic stimulation caused by assorted serum factors. On the day of the experiment the cell capsules are transferred to the microphysiometer and allowed to equilibrate in recording media (low buffer RPMI 1640, no bicarbonate, no serum (Molecular Devices Corporation, Sunnyvale, CA) containing 0.1% fatty acid free BSA), during which a baseline measurement of basal metabolic activity is established.

A standard recording protocol specifies a 100μl/min flow rate, with a 2 min total pump cycle which includes a 30 sec flow interrruption during which the acidification rate measurement is taken. Ligand challenges involve a 1 min 20 sec exposure to the sample just prior to the first post challenge rate measurement being taken, followed by two additional pump cycles for a total of 5 min 20 sec sample exposure. Typically, drugs in a primary screen are presented to the cells at 10 μM final concentration. Follow up experiments to examine dose-dependency of active compounds is then done by sequentially challenging he cells with a drug concentration range that exceeds the amount needed to generate responses ranging from threshold to maximal levels. Peptides included in the

microphysiometric screen included rat NPFF (FLFQPQRF-NH2) and rat A-18-F-amide (AGEGLSSPFWSLAAPQRF-NH2). Ligand samples are then washed out and the acidification rates reported are expressed as a percentage increase of the peak response over the baseline rate observed just prior to challenge.

Receptor/G protein co-transfection studies

A strategy for determining whether NPFF can couple preferentially to selected G proteins involves co-transfection of NPFF receptor cDNA into a host cell together with the cDNA for a G protein alpha sub-unit. Examples of G alpha sub-units include members of the Gαi/Gαo class (including Gαt2 and Gαz), the Gαq class, the Gαs class, and the Gα12/13 class. A typical procedure involves transient transfection into a host cell such as COS-7. Other host cells may be used. A key consideration is whether the cell has a downstream effector (a particular adenylate cyclase, phospholipase C, or channel isoform, for example) to support a functional response through the G protein under investigation. G protein beta gamma sub-units native to the cell are presumed to complete the G protein heterotrimer; otherwise specific beta and gamma sub-units may be co-transfected as well. Additionally, any individual or combination of alpha, beta, or gamma subunits may be co-transfected to optimize the functional signal mediated by the receptor.

The receptor/G alpha co-transfected cells are evaluated in a binding assay, in which case the radioligand binding may be enhanced by the presence of the optimal G protein coupling or in a functional assay designed to test the receptor/G protein hypothesis. In one example, the NPFF receptor may be hypothesized to inhibit cAMP accumulation

through coupling with G alpha sub-units of the Gαi/Gαo class. Host cells co-transfected with the NPFF receptor and appropriate G alpha sub-unit cDNA are stimulated with forskolin +/- NPFF agonist, as described above in cAMP methods. Intracellular cAMP is extracted for analysis by radioimmunoassay. Other assays may be substituted for cAMP inhibition, including $GTP\gamma^{35}S$ binding assays and inositol phosphate hydrolysis assays. Host cells transfected with NPFF minus G alpha or with G alpha minus NPFF would be tested simutaneously as negative controls. NPFF receptor expression in transfected cells may be confirmed in ^{125}I-NPFF protein binding studies using membranes from transfected cells. G alpha expression in transfected cells may be confirmed by Western blot analysis of membranes from transfected cells, using antibodies specific for the G protein of interest.

The efficiency of the transient transfection procedure is a critical factor for signal to noise in an inhibitory assay, much more so than in a stimulatory assay. If a positive signal present in all cells (such as forskolin-stimulated cAMP accumulation) is inhibited only in the fraction of cells successfully transfected with receptor and G alpha, the signal to noise ratio will be poor. One method for improving the signal to noise ratio is to create a stably transfected cell line in which 100% of the cells express both the receptor and the G alpha subunit. Another method involves transient co-transfection with a third cDNA for a G protein-coupled receptor which positively regulates the signal which is to be inhibited. If the co-transfected cells simultaneously express the stimulatory receptor, the inhibitory receptor, and a requisite G protein for the inhibitory receptor, then a positive signal may be elevated selectively in transfected

cells using a receptor-specific agonist. An example involves co-transfection of COS-7 cells with 5-HT4, NPFF1, and a G alpha sub-unit. Transfected cells are stimulated with a 5-HT4 agonist +/- NPFF1 protein. Cyclic AMP is expected to be elevated only in the cells also expressing NPFF1 and the G alpha subunit of interest, and a NPFF-dependent inhibition may be measured with an improved signal to noise ratio.

It is to be understood that the cell lines described herein are merely illustrative of the methods used to evaluate the binding and function of the mammalian receptors of the present invention, and that other suitable cells may be used in the assays described herein.

Electrophysiology

Methods for recording currents in Xenopus oocytes

Oocytes were harvested from *Xenopus laevis* and injected with mRNA transcripts as previously described (Quick and Lester, 1994; Smith et al.,1997). NPFF receptors and $G\alpha_{q/z}$ chimera synthetic RNA transcripts were synthesized using the T7 polymerase ("Message Machine," Ambion) from linearized plasmids or PCR products containing the complete coding region of the genes. Oocytes were injected with 10 ng NPFF receptors synthetic RNA and incubated for 3-8 days at 17 degrees. Three to eight hours prior to recording, oocytes were injected with 500 pg $G\alpha_{q/z}$ mRNA in order to observe coupling to Ca^{++} activated Cl^- currents. Dual electrode voltage clamp (Axon Instruments Inc.) was performed using 3 M KCl-filled glass microelectrodes having resistances of 1-2 Mohm. Unless otherwise specified, oocytes were voltage clamped at a holding potential of -80 mV. During recordings, oocytes were bathed in continuously flowing (1-3 ml/min) medium

containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5 (ND96). Drugs were applied either by local perfusion from a 10 ml glass capillary tube fixed at a distance of 0.5 mm from the oocyte, or by switching from a series of gravity fed perfusion lines.

Other oocytes may be injected with a mixture of receptor mRNAs and synthetic mRNA encoding the genes for G-protein-activated inward rectifiers (GIRK1 and GIRK4, U.S. Patent Nos. 5,734,021 and 5,728,535). Genes encoding G-protein inwardly rectifying K^+ (GIRK) channels 1 and 4 (GIRK1 and GIRK4) may be obtained by PCR using the published sequences (Kubo et al., 1993; Dascal et al., 1993; Krapivinsky et al., 1995 and 1995b) to derive appropriate 5' and 3' primers. Human heart cDNA may be used as template together with appropriate primers.

Heterologous expression of GPCRs in *Xenopus* oocytes has been widely used to determine the identity of signaling pathways activated by agonist stimulation (Gundersen et al., 1983; Takahashi et al., 1987). Activation of the phospholipase C (PLC) pathway is assayed by applying test compound in ND96 solution to oocytes previously injected with mRNA for the mammalian receptor and observing inward currents at a holding potential of -80 mV. The appearance of currents that reverse at -25 mV and display other properties of the Ca^{++}-activated Cl^- (chloride) channel is indicative of mammalian receptor-activation of PLC and release of IP3 and intracellular Ca^{++}. Such activity is exhibited by GPCRs that couple to G_q.

Measurement of inwardly rectifying K^+ (potassium) channel (GIRK) activity may be monitored in oocytes that have been co-injected with mRNAs encoding the mammalian receptor,

GIRK1, and GIRK4. The two GIRK gene products co-assemble to form a G-protein activated potassium channel known to be activated (i.e., stimulated) by a number of GPCRs that couple to G_i or G_o (Kubo et al., 1993; Dascal et al., 1993). Oocytes expressing the mammalian receptor plus the two GIRK subunits are tested for test compound responsivity by measuring K^+ currents in elevated ^+K solution containing 49 mM K^+. Activation of inwardly rectifying currents that are sensitive to 300 μM Ba^{++} signifies the mammalian receptor coupling to a G_i or G_o pathway in the oocytes.

Localization of mRNA coding for rat NPFF1 receptors

Development of probes for NPFF1: To facilitate the production of radiolabeled, antisense RNA probes a fragment of the gene encoding rat NPFF1 was subcloned into a plasmid vector containing RNA polymerase promotor sites. The full length cDNA encoding the rat NPFF1 was digested with Sph I (nucleotides 766-1111), and this 345 nucleotide fragment was cloned into the Sph I site of pGEM 3z, containing both sp6 and T7 RNA polymerase promotor sites. The construct was sequenced to confirm sequence identity and orientation. To synthesize antisense strands of RNA, this construct was linearized with Hind III and T7 RNA polymerase was used to incorporate radiolabeled nucleotide as described below.

A probe coding for the rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, a constitutively expressed protein, was used concurrently. GAPDH is expressed at a relatively constant level in most tissue and its detection is used to compare expression levels of the rat NPFF1 receptors genes in different regions.

Synthesis of probes: NPFF1 and GAPDH cDNA sequences preceded by phage polymerase promoter sequences were used to synthesize radiolabeled riboprobes. Conditions for the synthesis of riboprobes were: 0.25-1.0 μg linearized DNA plasmid template, 1.5 μl of ATP, GTP, UTP (10 mM each), 3 μl dithiothreitol (0.1M), 30 units RNAsin RNAse inhibitor, 0.5-1.0 μl (15-20 units/μl) RNA polymerase, 7.0 μl transcription buffer (Promega Corp.), and 12.5 μl α^{32}P-CTP (specific activity 3,000Ci/mmol). 0.1 mM CTP (0.02-1.0 μl) was added to the reactions, and the volumes were adjusted to 35 μl with DEPC-treated water. Labeling reactions were incubated at 37°C for 60 minutes, after which 3 units of RQ1 RNAse-free DNAse (Promega Corp.) were added to digest the template. Riboprobes were separated from unincorporated nucleotides using Microspin S-300 columns (Pharmacia Biotech). TCA precipitation and liquid scintillation spectrometry were used to measure the amount of label incorporated into the probe. A fraction of all riboprobes synthesized was size-fractionated on 0.25 mm thick 7M urea, 4.5% acrylamide sequencing gels. These gels were apposed to screens and the autoradiograph scanned using a phosphorimager (Molecular Dynamics) to confirm that the probes synthesized were full-length and not degraded.

Solution hybridization/ribonuclease protection assay (RPA): For solution hybridization 2.0 μg of mRNA isolated from tissues were used. Negative controls consisted of 30 μg transfer RNA (tRNA) or no tissue blanks. All mRNA samples were placed in 1.5 ml microfuge tubes and vacuum dried. Hybridization buffer (40 μl of 400 mM NaCl, 20 mM Tris, pH 6.4, 2 mM EDTA, in 80% formamide) containing 0.25-2.0 E^6 counts of each probe were added to each tube. Samples were heated at 95°C for 15 min, after which the

temperature was lowered to 55°C for hybridization.

After hybridization for 14-18 hr, the RNA/probe mixtures were digested with RNAse A (Sigma) and RNAse T1 (Life Technologies). A mixture of 2.0 μg RNAse A and 1000 units of RNAse T1 in a buffer containing 330 mM NACl, 10 mM Tris (pH 8.0) and 5 mM EDTA (400 μl) was added to each sample and incubated for 90 min at room temperature. After digestion with RNAses, 20 μl of 10% SDS and 50 μg proteinase K were added to each tube and incubated at 37°C for 15 min. Samples were extracted with phenol/chloroform:isoamyl alcohol and precipitated in 2 volumes of ethanol for 1 hr at -70°C. Pellet Paint (Novagen) was added to each tube (2.0 μg) as a carrier to facilitate precipitation. Following precipitation, samples were centrifuged, washed with cold 70% ethanol, and vacuum dried. Samples were dissolved in formamide loading buffer and size-fractionated on a urea/acrylamide sequencing gel (7.0 M urea, 4.5% acrylamide in Tris-borate-EDTA). Gels were dried and apposed to storage phosphor screens and scanned using a phosphorimager (Molecular Dynamics, Sunnydale, CA).

RT-PCR

For the detection of of low levels of RNA encoding rat NPFF1, RT-PCR was carried out on mRNA extracted from rat tissue. Reverse transcription and PCR reactions were carried out in 50 μl volumes using EzrTth DNA polymerase (Perkin Elmer). Primers with the following sequences were used:

RA Rsnorf2/NPFF F1:
CTCCTACTACCAACACTCCTCTCC (Seq. ID No. 39)

RA RSNORF2/NPFF1 B1:

ACGGGTTACGAGCATCCAG (Seq. ID No. 40)

These primers will amplify 490 base pair fragment from nucleotide 574 to 1064.

Each reaction contained 0.2 μg mRNA and 0.3μM of each primer. Concentrations of reagents in each reaction were: 300μM each of dGTP, dATP, dCTP, dTTP; 2.5mM $Mn(OAc)_2$; 50mM Bicine; 115 mM K acetate, 8% glycerol and 5 units EzrTth DNA polymerase. All reagents for PCR (except mRNA and oligonucleotide primers) were obtained from Perkin Elmer. Reactions were carried out under the following conditions: 65°C, 60 min; 94°C, 2 min; (94°C, 1 min; 65°C, 1 min) 35 cycles, 72°C, 10 min. PCR reactions were size fractionated by agarose gel electrophoresis usig 10% polyacrylamide. DNA was stained with SYBR Green I (Molecular Probes, Eugene, OR) and scanned on a Molecular Dynamics (Sunnyvale, CA) Storm 860 in blue flourescence mode at 450 nM.

Positive controls for PCR reactions consisted of amplification of the target sequence from a plasmid construct, as well as reverse transcribing and amplifying a known sequence. Negative controls consisted of mRNA blanks as well as primer blanks. To confirm that the mRNA was not contaminated with genomic RNA, samples were digested with RNAses before reverse transcription. Integrity of RNA was assessed by amplification of mRNA coding for GAPDH.

Localization of mRNA coding for NPFF-like receptors (hNPFF2)using RT-PCR

For the detection of low levels of RNA encoding hNPFF2 RT-PCR was carried out on mRNA extracted from tissue.

Reverse transcription and PCR reactions were carried out in 50 μl volumes using EzrTh DNA polymerase (Perkin Elmer). Primers with the following sequences were used:
JB 249: 5'-GATCAGTGGATTGGTCCAGGGAATATC-3' (SEQ. ID No. 41)
JB 250: 5'-CCAGGTAGATGTTGGCAAACAGCAC-3' (SEQ. ID No. 42)

These primers will amplify a 332 base pair fragment from TMIII to TMV.

Each reaction contained 0.1 ug mRNA and o.3uM of each primer. Concentrations of reagents in each reaction were 300uM each of dGTP, dATP, dCTP, dTTP, 2.5mM Mn(OAc)2, 50mM Bicine, 115 mM potassium acetate, 8% glycerol and 5 units EzrTth DNA polymerase. All reagents for PCR (except mRNA and oligonucleotide primers) were obtained from Perkin Elmer. Reactions were carried out under the following conditions: 65°C 60 min., 94°C 2 min, (94°C, 1 min, 65°C 1 min) 35 cycles, 72°C 10 min. PCR reactions were size fractionated by gel electrophoresis using 10% polyacrylamide. DNA was stained with SYBR Green I (Molecular Probes, Eugene OR) and scanned on a Molecular Dynamics (Sunnyvale, CA) Strom 860 in blue fluorescence mode at 450 nm.

Positive controls for PCR reactions consisted of amplification of the target sequence from a plasmid construct, as well as reverse transcribing and amplifying a known sequence. Negative controls consisted of mRNA blanks and primer blanks. To confirm that the mRNA was not contaminated with genomic DNA, samples were digested with RNAses before reverse transcription. Integrity of RNA was assessed by amplification of mRNA coding for GAPDH.

Results and Discussion

Cloning and Sequencing

rNPFF1 and hNPFF1

100 ng genomic DNA was subjected to MOPAC PCR with two degenerate primers designed based on the sixth and seventh transmembrane domains of over 180 receptors from the rhodopsin superfamily of G protein-coupled receptors. Two products from this reaction, MPR3-RGEN-31 and MPR3-RGEN-45 were found to be identical clones of a novel DNA sequence not found in the Genbank databases (Genembl, STS, EST, GSS), which had 30-40% amino acid identity with the known receptors dopamine D2, orexin 1, GALR1, angiotensin 1B and 5HT-2b. This novel clone was given the name SNORF2.

The full-length SNORF2 sequence was acquired by screening rat hypothalamic cDNA libraries by PCR using specific SNORF2 oligonucleotide primers. Pools of the rat hypothalamic cDNA library "I" were screened by PCR with SNORF2-specific primers JAB208 and JAB209. This screen yielded a positive pool I36. Successive PCR screening of sub-pools of this pool followed by high stringency hybridization of isolated colonies from the positive sub-pool I36-17 with the SNORF2-specific oligonucleotide probe indicated that the isolated clone I36E-17-1B-1 contained at least a partial clone of SNORF2. Sequencing of I36E-17-1B-1 revealed that this insert contained the coding region from the TMIII-TMIV loop through the stop codon, including some 3' untranslated sequence. From this sequence, a new forward primer, JAB221, was designed in TMV. PCR screening of a second rat hypothalamic cDNA library "J" with primers JAB221 and JAB209, and subsequent colony hybridization with the JAB211 probe on a low complexity positive sub-pool resulted in the isolation of a SNORF2 clone J-13-16-A1. This clone contained the full-

length coding sequence of SNORF2 (1296bp) with approximately 200 bp 5'untranslated sequence and 1.3 kb 3' untranslated sequence. The nucleotide sequence of SNORF2 and its translated amino acid sequence are represented in Figures 1 and 2, respectively. As shown in Figure 1, SNORF2 contains two potential initiating methionines upstream of TMI.

Hydophobicity (Kyte-Doolittle) analysis of the amino acid sequence of the full-length clone indicates the presence of seven hydrophobic regions, which is consistent with the seven transmembrane domains of a G protein-coupled receptor. The seven expected transmembrane domains are mapped out in Figure 3. A comparison of nucleotide and peptide sequences of SNORF2 with sequences contained in the Genbank/EMBL/SwissProtPlus databases reveals that the amino acid sequence of this clone is most related to the orexin 1 and 2 receptors (45% and 40% identity, respectively) as well as the neuropeptide Y receptors Y1, Y2 and Y4 (~30% identity). Further homology analysis of SNORF2 against the Synaptic Pharmaceutical Corporation in-house database revealed that SNORF2 has a very high degree of identity with a proprietary Synaptic Pharmaceutical Corporation human partial GPCR clone named PLC29b (85% nucleotide identity, 93% amino acid identity). PLC29b was originally isolated from a human genomic library using oligonucleotide probes for NPY4, and includes part of the amino terminus and TMs I to IV. Partial nucleotide and amino acid sequence of PLC29b (human SNORF2) is represented in Figures 4 and 5, respectively. Based on sequence similarity, PLC29b appears to be a partial clone of the human homologue of SNORF2. Therefore, this human homolog of SNORF2 has been named hNPFF1. A GAP alignment demonstrating the high homology between these species

homologues is represented in Figure 6.

SNORF2 has several potential protein kinase C (PKC) phosphorylation motifs throughout its amino acid sequence: threonine 154 in the second intracellular loop, threonine 263 and serine 264 in the third intracellular loop, and serine 363 in the intracellular carboxy-terminal tail. It also has four potential N-linked glycosylation sites at asparagines 10 and 18 in the amino-terminal tail and at asparagines 113 and 195 in the first and second extracellular loops, respectively.

hNPFF2

In analyzing the sequence of rNPFF1 and its homology to other sequences in GenBank, a 532 bp EST with the accession number AA449919 was identified which had a high degree of identity to rNPFF1. The translation of this sequence indicated that it coded for the region between the first extracellular loop and the beginning of the sixth transmembrane domain of a G protein-coupled receptor (GPCR). Although AA449919 was documented as being similar to the *Drosophila melanogaster* NPY receptor (accession number P25931), it was found that the amino acid sequence encoded by this EST was much more similar to NPFF1. The predicted amino acid sequence of AA449919 and rNPFF1 are 50% identical, while the amino acid sequence of the *Drosophila* NPY receptor is only 31% identical to the translation of AA449919. Because of the high degree of identity between AA449919 and rNPFF1, AA449919 was given the name hNPFF2, representing a member of a novel family of NPFF receptors of which there is currently only one member, NPFF1.

The full length sequence of NPFF-like (hNPFF2) was

acquired by 5'/3' RACE using human spleen cDNA as a template, as described above, demonstrating that the coding region of hNPFF-like (hNPFF2) is 1260 bp, coding for a protein of 420 amino acids. Sequencing of clones from several independent PCR reactions using spleen, heart, and spinal cord cDNA as templates and subsequent alignment of these clones with Sequencher 3.0 was used to confirm the sequence of hNPFF-like (hNPFF2). The full-length nucleotide sequence of human NPFF2 is shown in Figure 7, and its translated amino acid sequence is shown in Figure 8. The seven putative transmembrane domains of hNPFF-like (hNPFF2) are defined in Figure 9.

Like the original EST AA449919, the amino acid sequence encoded by the full-length DNA sequence of hNPFF2 is most similar to rNPFF1 (48% identity), as shown in the GAP alignment between the two receptors in Figure 10. The next-best matches in SWPLUS to full-length hNPFF2 are the *Drosophila* NPYR (accession number P25931, 34% identity) and TLR2 (accession number P30975, 32% identity), human orexin 1 and 2 receptors (O43613, 31% and O43614, 29%, respectively) and human NPY1 and Y4 receptors (P25929, 31% and P50391, 32%, respectively). A Blast search of the EST database using the full-length nucleotide sequence of hNPFF2 revealed an EST (Accession number AA449920) that is identical to hNPFF2 from the end of TM7 through the stop codon. ESTs AA44919 and AA44920 are the same clone sequenced from 5' end or the 3' end, respectively.

hNPFF2 contains several potential N-linked glycosylation sites. The first three sites, asparagines 8, 20, and 31 are in the N-terminal extracellular domain. Another potential N-linked glycosylation site, at position 198, is in the second extracellular loop. This receptor also

contains one potential PKC phosphorylation site at threonine 156 in the second intracellular loop, and two potential PKC phosphorylation sites in the third intracellular loop at threonine 254 and serine 266.

hNPFF1

The sequence of hNPFF1 from the initiating methionine to TMIV was determined to be present in a partial clone, plc29b, found in a Synaptic Pharmaceutical Corporation in-house database. Additional sequence, including TMIV through the stop codon, was determined by sequencing a vector-anchored PCR product from a human cosmid library clone identified by hybridization with a ^{32}P-labeled probe (BB609) corresponding to the II/III loop of plc29b. Next, a human spinal cord library was screened by PCR using primers designed against the partial hNPFF1 sequence, BB729 and BB728. One positive pool, W4, was subdivided and a positive sub-pool was screened by colony hybridization with a ^{32}P-labeled probe from TMII of hNPFF1, BB676. Plasmid DNA was isolated for clone W4-18-4, renamed BO98, and DNA sequencing revealed that it was full-length but in the wrong orientation for expression in the expression vector pEXJ. To obtain a full-length hNPFF1 construct in the correct orientation, BO98 was amplified with BB757 and BB758, and the resulting product ligated into pcDNA3.1 and transformed into DH5α cells. The sequence of one of these transformants was identical to the hNPFF1 sequence previously determined from the consensus of BO98 and the two cosmid clones. This human NPFF1 construct in pcDNA3.1 in the correct orientation was renamed BO102.

The hNPFF1 clone contains an open reading frame with 1293 nucleotides and predicts a protein of 430 amino acids (Figures 11 and 12). Seven transmembrane domains predicted

by hydrophobicity analysis are indicated in Figure 13. The sequence of hNPFF1 was determined to be most similar to the rat NPFF1 (86% nucleotide identity, 87% amino acid identity) and human NPFF2 (56% nucleotide identity, 49% amino acid identity (Figure 14)). The human NPFF1 receptor also shares homology with human orexin$_1$ (53% nucleotide identity, 35% amino acid identity), human orexin$_2$ (43% nucleotide identity, 33% amino acid identity), human NPY$_2$ (47% nucleotide identity, 31% amino acid identity), human CCK$_A$ (46% nucleotide identity, 32% amino acid identity), and human CCK$_B$ (46% nucleotide identity, 26% amino acid identity).

Electrophysiology

NPFF1

Oocytes injected with both SNORF2 and chimeric G$\alpha_{q/z}$ synthetic RNAs generated robust inward currents in response to NPFF and the related peptide A-18-F-amide at 1 μM (Figure 15A,B). Control oocytes receiving only G-protein synthetic RNA were unresponsive to these peptides. Responses to NPFF were concentration-dependent with a threshold for activation of inward current at 30 nM. The C-terminal tetrapeptide PQRF-amide also elicited responses at a concentration of 10 μM (Figure 15C). Analogs of NPFF containing a tyrosine residue at the N-terminus or internally including Y-8-F-amide, [tyr^9]A-18-F-amide and Y-18-F-amide also displayed activity at 1 μM. Unrelated neuropeptides and other neurotransmitters including melanin concentrating hormone, orexin B, PYY, 5-HT, nociceptin, galanin and CCK failed to activate oocytes injected with the SNORF2 synthetic RNA. The functional responsiveness to NPFF and related peptides strongly suggests that SNORF2 encodes a receptor for neuropeptide FF (NPFF); therefore SNORF2 was renamed NPFF1. Similarly,

SNORF2-like was renamed NPFF-like.

Oocytes injected with NPFF1 and not the chimeric G-protein synthetic RNA failed to generated responses to NPFF. This observation supports the hypothesis that NPFF1 couples to G-proteins of the $G\alpha_i/G\alpha_o/G\alpha_z$ class, and by virtue of the N-terminal portion of $G\alpha_{q/z}$, subsequently activates phospholipase C. In oocytes expressing both NPFF1 and $G\alpha_{q/z}$, Cl^- currents were abolished by prior injection of 10 mM EGTA, demonstrating the Ca^{++} dependence of these currents.

NPFF2

Oocytes injected with both the NPFF-like PCR product and chimeric $G\alpha_{q/z}$ synthetic RNAs generated large inward currents in response to 1 μM NPFF (Figure 16A). A-18-F-amide and PQRF-amide also at 1 μM activated similar inward currents, although the magnitude of currents generated by PGRF-amide were smaller. No activity was observed using FMRF-amide at 1 μM. The unrelated neuropeptides orexin A, NPY, galanin, and neurokinin A at 1 μM also failed to activate responses in oocytes injected with NPFF-like mRNA (Figure 16B). Oocytes injected with both the NPFF-like plasmid (BO89) and chimeric $G\alpha_{q/z}$ synthetic RNAs also produced robust currents in response to NPFF (Figure 16C). Based on these results, NPFF-like was renamed NPFF2. Oocytes injected with NPFF2 and not chimeric G-protein mRNA failed to generate responses to NPFF. This observation supports the hypothesis that NPFF2 couples to G-proteins of the $G\alpha_i/G\alpha_o/G\alpha_z$ class, and by virtue of the N-terminal portion of $G\alpha_{q/z}$, subsequently activates phospholipase C.

Microphysiometry

CHO cells transiently expressing either NPFF1 alone or NPFF1 accompanied by the chimeric protein Gq/Gz produced robust increases in metabolism when exposed to either NPFF or the related peptide A-18-F-amide as evidenced by increased rates of extracellular acidification when measured by the microphysiometric technique (Figures 17A and 17B). Whereas control cells, not expressing NPFF1, produced no increase in acidification rates to either NPFF or A-18-F-amide. In all cases the NPFF1 mediated responses were dose-dependent. CHO cells transfected with NPFF1 alone produced an EC50 value of 19.3 nM for NPFF while cells transfected NPFF1 and the chimeric Gz/Gq produced an EC50 of 27.7 nM for NPFF. Challenges with A-18-F-amide were conducted only on cells that had been transfected with NPFF1 alone. These cells produced an EC50 value of 150nM for A-18-F-amide. The functional responsiveness to NPFF and A-18-F-amide supports the notion that NPFF1 encodes a receptor for neuropeptide FF (NPFF).

Radioligand binding assays

Cos-7 cells transiently expressing the gene encoding the novel rat NPFF1 receptor were used for pharmacological evaluation. Membranes harvested from transiently transfected Cos-7 cells exhibited high affinity, saturable [^{125}I]D-Tyr-NPFF ([D-Tyr^{1}(NMe)Phe^{3}]NPFF) binding. Nonlinear analysis of [^{125}I]D-Tyr-NPFF saturation data yielded an equilibrium dissociation constant (K_d) of 0.335 ± 0.045 nM and a binding site density (B_{max}) of 180 ± 11 fmol/mg protein. Specific [^{125}I]D-Tyr-NPFF binding was about 50% of total binding at a radioligand concentration equal to the K_d value. Mock-transfected host cells did not display specific [^{125}I]D-Tyr-NPFF binding.

To further assess the pharmacological identity of the newly isolated NPFF1 receptor gene, detailed binding properties of cloned NPFF1 receptor were determined from nonlinear analysis of competition of high affinity [^{125}I]D-Tyr-NPFF binding. The rank order of affinity of compounds to displace specific [^{125}I]D-Tyr-NPFF binding is shown in Table 1.

The binding profile of rat NPFF1 was compared to that of rat spinal cord membranes. Interestingly some differences were observed in the pharmacological profile between the two preparations. (See * Table 2). Notably, frog NPFF did not displace the binding on the NPFF1 receptor up to 1 uM whereas it displayed a high affinity at the rat spinal cord. Furthermore, several compounds displayed significantly different affinities between NPFF1 receptor and the spinal cord membranes. These compounds are highlighted in Table 1 and are ([^{125}I]D-Tyr-NPFF, A18Famide, Y8Famide, [Y^{9}]A18Famide, Dynorphin A 1-13, Neuropeptide F and Met-Enk-NH2. These data indicate the presence of additional NPFF receptor subtypes on the rat spinal cord.

The ability of NPFF1 receptors to functionally couple to PI was tested using intact Cos-7 cells transiently expressing NPFF1. Full dose-response curves were determined for NPFF-mediated total IP release (Figure 18A). NPFF stimulated total IP release with an EC50 of 23 nM and an Emax of approximately 200% basal. This weak stimulation was most probably mediated by NPFF1 coupling to a Gi/Go G-protein via βγ-induced PI turnover, since the response was abolished by pretreatment with pertussis toxin but not cholera toxin. In contrast, a robust

stimulation of total IP release was observed following NPFF in Cos-7 cells transfected with both the NPFF1 receptor and the Gq/Gz chimera (Figure 18B). NPFF stimulated total IP release with an EC50 of 2.95 nM, and an Emax of approximately 1500% basal. As anticipated, neither PTX nor CTX attenuated this response. Similar to what was observed in oocytes, this suggests a coupling in Cos-7 cells to G-proteins of the Gαi/Gαo/Gαz class.

Table 1

pKi for cloned rat NPFF1 receptor binding in COS-7 cells

COMPOUND	MEAN	SEM	n
NPFF(F-8-Fa)	8.535	0.02	2
(D-Tyr^{1}-(NMe)Phe^{3})NPFF	8.549	0.13	4
A18Fa	7.495	0.11	2
PQRFa	8.182	0.03	2
FMRFa	8.481	0.05	2
YFa	8.382	0.22	2
[Y^{9}]A18Fa	7.558	0.12	2
hPP	5.000	0.00	2
fPP	5.500	0.35	2
substance P	5.000	0.00	2
Dynorphin A1-13	6.838	0.29	2
(3D)Y8Fa	8.623	0.44	4
(2D)Y8Fa	8.330	0.15	4
CCK8	5.000	0.00	2
galanin	5.000	0.00	2
dopamine	5.000	0.00	2
naloxone	5.000	0.00	2
CGRP	5.000	0.00	2
AF-1	6.634	0.13	2
AF-2	7.023	0.41	2
SchistFLRF	5.960	0.68	2
Met5-Arg6-Phe7-Enk-NH2	7.350	0.22	4
Met5-Arg6-Phe7-Enk-OH	5.000	0.00	2
Neuropeptide F	6.110	0.06	4

desamino-nor-Y8Ra	7.270	0.10	3
(2DME)Y8Fa	9.200	0.01	3
L-arginine	5.000	0.00	1
D-arginine	5.000	0.00	1
desipramine	5.000	0.00	1
fenfluramine	5.000	0.00	1
harmine	5.000	0.00	1
levocabastine	5.000	0.00	1
ibogaine	5.000	0.00	1
ritanserine	5.000	0.00	1
a-MSH	5.000	0.00	1
Tyr-MIF-1	5.000	0.00	1
nociceptin	5.000	0.00	1
nocistatin	5.000	0.00	1
PMRFa	8.550	0.06	2
FTRF	7.870	0.10	2
FFRF	8.000	0.001	2

Table 2

pKi for rat spinal cord membrane receptor binding

COMPOUND	MEAN	SEM	n
NPFF(F-8-fa)	9.055	0.08	2
(D-Tyr^1-(NMe)Phe^3)NPFF	*9.724	0.25	4
A18Fa	*9.000	0.21	2
PQRFa	8.541	0.07	2
FMRFa	8.493	0.23	2
Y8Fa	*9.189	0.06	2
[Y^9]A18Fa	*8.502	0.01	2
hPP	5.000	0.00	3
fPP	*9.118	0.06	3
substance P	5.000	0.00	1
Dynorphin A1-13	*5.700	0.50	2
(3D)Y8Fa	9.123	0.12	4
(2D)Y8Fa	*9.212	0.23	4
CCK8	5.000	0.00	2
galanin	5.000	0.00	2
dopamine	5.000	0.00	2
naloxone	5.000	0.00	2
CGRP	5.000	0.00	2
AF-1	*7.563	0.47	2
AF-2	*7.965	0.24	2
SchistFLRF	6.390	0.23	2
Met-Enk-NH2	*8.400	0.08	4
Met-Enk-OH	5.000	0.00	2
Neuropeptide F	*8.100	0.10	3
desamino-nor-Y8Ra	7.51	0.07	3

(2DME)Y8Fa	9.570	0.30	4
L-arginine	5.000	0.00	1
D-arginine	5.000	0.00	1
desipiramine	5.000	0.00	1
fenfluramine	5.000	0.00	1
harmine	5.000	0.00	1
levocabastine	5.000	0.00	1
ibogaine	5.000	0.00	1
ritanserine	5.000	0.00	1
a-MSH	5.000	0.00	1
Tyr-MIF-1	5.000	0.00	1
nociceptin	5.000	0.00	1
nocistatin	5.000	0.00	1
PMRFa	9.370	0.11	2
FTRF	8.160	0.16	2
FFRF	8.980	0.001	2

AF-1 = FMRF-like peptide H_2N-Lys-Asn-Gln-Phe-Ile-Arg-Phe-NH_2

AF-2 H-Lys-His-Gln-Tyr-Leu-Arg-Phe-NH_2

Schisto(FLRFNH_2) = Pro-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-amide

Met^5, Arg^6, Phe^7- NH_2 = enhephalinamide

Met^5, Arg^6, Phe^7- OH = enhephalin

Localization

Detection of mRNA coding for rat NPFF1 receptors: mRNA was isolated from multiple tissues (Table 3) and assayed as described. The distribution of mRNA encoding rat NPFF1 receptors is widespread throughout the central nervous system, and structures associated with the nervous system (Table 3, Figures 19, 20). The highest levels of rNPFF1 mRNA are found in the hypothalamus and the pituitary gland. The protected segment seen with mRNA isolated from the pituitary, adrenal gland and ovary is considerably shorter than that seen in other tissue (Figure 20) and indicates the possibility of splice variants of this receptor. Peripheral organs contain little or no mRNA encoding rNPFF1 with the exception of the testes, ovary, the adrenal medulla and the adrenal cortex. There is good correlation between the distribution determined by RT-PCR and RPA (Table 3, Figures 19, 20). RT-PCR detected rat NPFF1 in more areas than RPA as it is a more sensitive technique.

High levels of mRNA encoding NPFF receptors in the hypothalamus and pituitary, with relatively low expression in most of the other regions assayed implicates this receptor in neuroendocrine control, as well as the control of feeding and metabolic regulation. Its presence in other areas, including the spinal cord, medulla and dorsal root ganglia implicate NPFF receptors as a potential modulator of pain and/or sensory transmission. Low levels in the hippocampal formation indicate a possible role in learning and memory.

Table 3
Summary of distribution of mRNA coding for rat NPFF1 receptors

Tissue	RT-PCR	Ribonuclease protection assay (RPA)	Potential applications
adrenal cortex	+	+	regulation of steroid hormones
adrenal medulla	+	++	regulation of epinephrine release
urinary bladder	-	-	urinary incontinence
duodenum	+/-	-	gastrointestinal disorders
heart	+/-	-	cardiovascular indications
kidney	+	-	electrolyte balance, hypertension
liver	+/-	-	diabetes
lung	+/-	-	respiratory disorders, asthma
ovary	+	+	reproductive function
pancreas	+/-	NA	diabetes, endocrine disorders
spleen	+/-	-	immune disorders
stomach	+/-	-	gastrointestinal disorders

striated muscle	+/-	-	musculoskeletal disorders
testicle	+/-	+	reproductive function
uterus	+/-	-	reproductive function
vas deferens	-	-	reproductive function
whole brain	+++		
spinal cord	++	++	analgesia, sensory modulation and transmission
amygdala	+++	+/-	
caudate/putamen	++	-	modulation of dopaminergic function
cerebellum	+++	+	motor coordination
cerebral cortex	++	+	Sensory and motor integration, cognition
DRG	+	+	sensory transmission
hippocampus	+++	+	cognition/memory
hypothalamus	+++	+++	appetite/obesity, neuroendocrine regulation
medulla	++	++	analgesia, motor coordination
olfactory bulb	++	NA	olfaction
pituitary	+++	+++	Endocrine/neuroendocrine regulation

substantia nigra	+++	++	Modulation of dopaminergic function
superior cervical ganglion	+	-	modulation of sympathetic innervation

NA = not assayed

Localization of mRNA coding for hNPFF2 receptors using RT-PCR

Detection of mRNA coding for hNPFF2 receptors

mRNA was isolated from multiple tissues (Table 4) and assayed as described. The distribution of mRNA encoding hNPFF2 receptors is widespread throughout all regions assayed. (Table 4, Figure 21).

Table 4

Distribution of mRNA coding for hNPFF2 receptors

Region	hNPFF2	Potential Implications
liver	++	Diabetes
kidney	++	Hypertension, electrolyte balance
Lung	++	Respiratory disorders, asthma
heart	++	Cardiovascular indications
stomach	++	Gastrointestinal disorders
small intestine	++	Gastrointestinal disorders
spleen	++	Immune function
pancreas	++	Diabetes, endocrine disorders
striated muscle	++	Musculoskeletal disorders
pituitary	++	Endocrine/neuroendocrine regulation
whole brain	++	
amygdala	++	Depression, anxiety, mood disorders
hippocampus	++	Cognition/memory
spinal cord	++	Analgesia, sensory modulation and transmission
cerebellum	++	Motor coordination
thalamus	++	sensory integration
substantia nigra	++	Modulation of dopaminergic function and motor coordination
caudate	++	Modulation of dopaminergic function

fetal brain	++	Developmental disorders
fetal lung	++	Developmental disorders
fetal kidney	++	Developmental disorders
fetal liver	++	Developmental disorders
HEK-293 cells	+	
HeLa cells	-	
Jurkat cells	-	

The cloning of the gene encoding NPFF receptors has provided the means to explore their physiological roles by pharmacological characterization, and by Northern and *in situ* mapping of its mRNA distribution. Further, the availability of the DNA encoding the NPFF receptors will facilitate the development of antibodies and antisense technologies useful in defining the functions of the gene products *in vivo*. Antisense oligonucleotides which target mRNA molecules to selectively block translation of the gene products *in vivo* have been used successfully to relate the expression of a single gene with its functional sequelae. Thus, the cloning of these receptor genes provides the means to explore their physiological roles in the nervous system and elsewhere, and may thereby help to elucidate structure/function relationships within the GPCR superfamily.

REFERENCES

1. Yang, H.Y., Fratta, W., Majane, E.A., and Costa, E. Isolation, sequencing, synthesis, and pharmacological characterization of two brain neuropeptides that modulate the action of morphine. *Proc.Natl.Acad.Sci.U.S.A.* 82(22):7757-7761, 1985.

2. Vilim, E.S., Ziff, E. Cloning of the neuropeptide NPFF and NPAF precursor form bovine, rat, mouse, and human. Soc. Neurosci. 21:760 (1995).

3. Panula, P., Aarnisalo, A.A., and Wasowicz, K. Neuropeptide FF, a mammalian neuropeptide with multiple functions [published erratum appears in Prog. Neurobiol. 1996 Jun; 49(3):285]. *Prog. Neurobiol.*. 48(4-5):461-487, 1996.

4. Roumy, M. and Zajac, J.M. Neuropeptide FF, pain and analgesia. *Eur.J.Pharmacol.* 345(1):1-11, 1998.

5. Payza, K., Akar, C.A., and Yang, H.Y. Neuropeptide FF receptors: structure-activity relationship and effect of morphine. *J.Pharmacol.Exp.Ther.* 267(1):88-94, 1993.

6. Raffa, R.B., Kim, A., Rice, K.C., de Costa, B.R., Codd, E.E., and Rothman, R.B. Low affinity of FMRFamide and four FaRPs (FMRFamide-related peptides), including the mammalian-derived FaRPs F-8-Famide (NPFF) and A-18-Famide, for opioid mu, delta, kappa 1, kappa 2a, or kappa 2b receptors.

Peptides 15(3):401-404, 1994.

7. Malin, D.H., Lake, J.R., Arcangeli, K.R., Deshotel, K.D., Hausam, D.D., Witherspoon, W.E., Carter, V.A., Yang, H.Y., Pal, B., and Burgess, K. Subcutaneous injection of an analog of neuropeptide FF precipitates morphine abstinence syndrome. *Life Sci.* 53(17):PL261-6, 1993.

8. Malin, D.H., Lake, J.R., Fowler, D.E., Hammond, M.V., Brown, S.L., Leyva, J.E., Prasco, P.E., and Dougherty, T.M. FMRF-NH2-like mammalian peptide precipitates opiate-withdrawal syndrome in the rat. *Peptides* 11(2):277-280, 1990.

9. Malin, D.H., Lake, J.R., Leyva, J.E., Hammond, M.V., Rogillio, R.B., Arcangeli, K.R., Ludgate, K., Moore, G.M., and Payza, K. Analog of neuropeptide FF attenuates morphine abstinence syndrome. *Peptides* 12(5):1011-1014, 1991.

10. Lake, J.R., Hebert, K.M., Payza, K., Deshotel, K.D., Hausam, D.D., Witherspoon, W.E., Arcangeli, K.A., and Malin, D.H. Analog of neuropeptide FF attenuates morphine tolerance. *Neurosci.Lett.* 146(2):203-206, 1992.

11. Lake, J.R., Hammond, M.V., Shaddox, R.C., Hunsicker, L.M., Yang, H.Y., and Malin, D.H. IgG from neuropeptide FF antiserum reverses morphine tolerance in the rat. *Neurosci.Lett.* 132(1):29-32, 1991.

12. Malin, D.H., Lake, J.R., Smith, D.A., Jones,

J.A., Morel, J., Claunch, A.E., Stevens, P.A., Payza, K., Ho, K.K., and Liu, J. Subcutaneous injection of an analog of neuropeptide FF prevents naloxone-precipitated morphine abstinence syndrome. *Drug Alcohol Depend.* 40(1):37-42, 1995.

13. Altier, N. and Stewart, J. Neuropeptide FF in the VTA blocks the analgesic effects of both intra-VTA morphine and exposure to stress. *Brain Res.* 758(1-2):250-254, 1997.

14. Oberling, P., Stinus, L., Le Moal, M., and Simonnet, G. Biphasic effect on nociception and antiopiate activity of the neuropeptide FF (FLFQPQRFamide) in the rat. *Peptides* 14(5):919-924, 1993.

15. Kavaliers, M. Inhibitory influences of mammalian FMRFamide (Phe-Met-Arg-Phe-amide)-related peptides on nociception and morphine- and stress-induced analgesia in mice. *Neurosci.Lett.* 115(2-3):307-312, 1990.

16. Kavaliers, M. and Yang, H.Y. IgG from antiserum against endogenous mammalian FMRF-NH2-related peptides augments morphine- and stress-induced analgesia in mice. *Peptides* 10(4):741-745, 1989.

17. Kavaliers, M. Innes, D. Sex differences in the effects of neuropeptide FF and IgG from neuropeptide FF on morphine- and stress-induced analgesia. Peptides 13(3):603-607, 1992.

18. Gicquel, S., Mazarguil, H., Allard, M., Simonnet, G., and Zajac, J.M. Analogues of F8Famide resistant to degradation, with high affinity and in vivo effects. *Eur.J.Pharmacol.* 222(1):61-67, 1992.

19. Gouarderes, C., Sutak, M., Zajac, J.M., and Jhamandas, K. Antinociceptive effects of intrathecally administered F8Famide and FMRFamide in the rat. *Eur.J.Pharmacol.* 237(1):73-81, 1993.

20. Gouarderes, C., Jhamandas, K., Sutak, M., and Zajac, J.M. Role of opioid receptors in the spinal antinociceptive effects of neuropeptide FF analogues. *Br.J.Pharmacol.* 117(3):493-501, 1996.

21. Lee, C.H., Wasowicz, K., Brown, R., Majane, E.A., Yang, H.T., and Panula, P. Distribution and characterization of neuropeptide FF-like immunoreactivity in the rat nervous system with a monoclonal antibody. *Eur.J.Neurosci.* 5(10):1339-1348, 1993.

22. Kivipelto, L. Ultrastructural localization of neuropeptide FF, a new neuropeptide in the brain and pituitary of rats. *Regul.Pept.* 34(3):211-224, 1991.

23. Kivipelto, L. and Panula, P. Central neuronal pathways containing FLFQPQRFamide-like (morphine-modulating) peptides in the rat brain. *Neuroscience* 41(1):137-148, 1991.

24. Allard, M., Labrouche, S., Nosjean, A., and

Laguzzi, R. Mechanisms underlying the cardiovascular responses to peripheral administration of NPFF in the rat. *J.Pharmacol.Exp.Ther.* 274(1):577-583, 1995.

25. Laguzzi, R., Nosjean, A., Mazarguil, H., and Allard, M. Cardiovascular effects induced by the stimulation of neuropeptide FF receptors in the dorsal vagal complex: An autoradiographic and pharmacological study in the rat. *Brain Res.* 711(1-2):193-202, 1996.

26. Kivipelto, L., Aarnisalo, A., and Panula, P. Neuropeptide FF is colocalized with catecholamine-synthesizing enzymes in neurons of the nucleus of the solitary tract. *Neurosci.Lett.* 143(1-2):190-194, 1992.

27. Panula, P., Kivipelto, L., Nieminen, O., Majane, E.A., and Yang, H.Y. Neuroanatomy of morphine-modulating peptides. *Med.Biol.* 65(2-3):127-135, 1987.

28. Allard, M., Geoffre, S., Legendre, P., Vincent, J.D., and Simonnet, G. Characterization of rat spinal cord receptors to FLFQPQRFamide, a mammalian morphine modulating peptide: a binding study. *Brain Res.* 500(1-2):169-176, 1989.

29. Allard, M., Zajac, J.M., and Simonnet, G. Autoradiographic distribution of receptors to FLFQPQRFamide, a morphine-modulating peptide, in rat central nervous system. *Neuroscience* 49(1):101-116, 1992.

30. Gouarderes, C., Tafani, J.A.M., and Zajac, J.M. Affinity of neuropeptide FF analogs to opioid receptors in the rat spinal cord. *Peptides* 19(4):727-730, 1998.

31. Payza, K. and Yang, H.Y. Modulation of neuropeptide FF receptors by guanine nucleotides and cations in membranes of rat brain and spinal cord. *J.Neurochem.* 60(5):1894-1899, 1993.

32. Devillers, J.P., Mazarguil, H., Allard, M., Dickenson, A.H., Zajac, J.M., and Simonnet, G. Characterization of a potent agonist for NPFF receptors: binding study on rat spinal cord membranes. *Neuropharmacology* 33(5):661-669, 1994.

33. Gicquel, S., Mazarguil, H., Desprat, C., Allard, M., Devillers, J.P., Simonnet, G., and Zajac, J.M. Structure-activity study of neuropeptide FF: contribution of N-terminal regions to affinity and activity. *J.Med.Chem.* 37(21):3477-3481, 1994.

34. Dupouy, V. and Zajac, J.M. Neuropeptide FF receptors in rat brain: A quantitative light-microscopic autoradiographic study using [125I][D.Tyr1, (NMe)Phe3]NPFF. *Synapse* 24(3):282-296, 1996.

35. Gouarderes, C., Tafani, J.A.M., Mazarguil, H., and Zajac, J.M. Autoradiographic characterization of rat spinal neuropeptide FF receptors by using [125I][D.Tyr1, (NMe)Phe3]NPFF. *Brain Res.Bull.* 42(3):231-238, 1997.

36. Gherardi, N. and Zajac, J.M. Neuropeptide FF receptors of mouse olfactory bulb: Binding properties and stimulation of adenylate cyclase activity. *Peptides* 18(4):577-583, 1997.

37. Kontinen, V.K., Aarnisalo, A.A., Idaenpaeaen-Heikkilae, J.J., Panula, P., and Kalso, E. Neuropeptide FF in the rat spinal cord during carrageenan inflammation. *Peptides* 18(2):287-292, 1997.

38. Wei, H., Panula, P., and Pertovaara, A. A differential modulation of allodynia, hyperalgesia and nociception by neuropeptide FF in the periaqueductal gray of neuropathic rats: Interactions with morphine and naloxone. *Neuroscience* 86(1):311-319, 1998.

39. Robert, J.J., Orosco, M., Rouch, C., Jacquot, C., and Cohen, Y. Unexpected responses of the obese "cafeteria" rat to the peptide FMRF-amide. *Pharmacol.Biochem.Behav.* 34(2):341-344, 1989.

40. Murase, T., Arima, H., Kondo, K., and Oiso, Y. Neuropeptide FF reduces food intake in rats. *Peptides* 17(2):353-354, 1996.

41. Kavaliers, M., Hirst, M., and Mathers, A. Inhibitory influences of FMRFamide on morphine- and deprivation-induced feeding. *Neuroendocrinology*. 40(6):533-535, 1985.

42. Muthal, A.V., Mandhane, S.N., and Chopde, C.T. Central administration of FMRFamide produces

antipsychotic-like effects in rodents. *Neuropeptides* 31(4):319-322, 1997.

43. Malin, D.H., Lake, J.R., Short, P.E., Blossman, J.B., Lawless, B.A., Schopen, C.K., Sailer, E.E., Burgess, K., and Wilson, O.B. Nicotine abstinence syndrome precipitated by an analog of neuropeptide FF. *Pharmacol.Biochem.Behav.* 54(3):581-585, 1996.

44. U. S. Patent No. 5,602,024 (Gerald et al. Feb. 11, 1997).

45. Coleman, A. (1984) Transcription and Translation: A Practical Approach (B.D. Hanes, S.J. Higgins, eds., pp 271-302, IRL Press, Oxford, 1984)

46. Dascal, N., Schreibmayer, W., Lim, N. F., Wang, W., Chavkin, C., DiMagno, L., Labarca, C., Kieffer, B.L., Gaveriaux-Ruff, C., Trollinger, D., Lester, H. A., Davidson, N. (1993) Proc. Natl. Acad. Sci. USA 90:10235-10239.

47. Fargin, A.; Raymond, J. R.; Lohse, M. J.; Kobilka, B. K.; Caron, M. G.; Lefkowitz, R. J. Nature 335:358-360 (1988).

48. Fong, T.M.; Huang, R.C.; Yu, H.; Swain, C.J.; Underwood, D.; Cascieri, M.A.; Strader, C.D. Can. J. Physiol. Pharmacol. 73(7):860-865 (Jul 1995).

49. Graziano, M.P.; Hey, P.J.; Strader, C.D. Receptors Channels 4(1):9-17 (1996).

50. Guam, X.M.; Amend, A.; Strader, C.D.; Mol. Pharmacol. 48(3):492-498 (Sep 1995).

51. Krapivinsky, G., Gordon, E. A., Wickman B., Velimirovic, B., Krapivinsky, L., Clapham, D. E. (1995) Nature 374:135-141.

52. Krapivinsky, G., Krapivinsky, L., Velimirovic, B., Wickman, K., Navarro, B., Clapham, D. E., (1995b) J. Biol. Chem. 270:28777-28779.

53. Kubo, Y., Reuveny, E., Slesinger, P. A., Jan, Y. N., Jan, L.Y. (1993) Nature 364:802-806.

54. Masu, Y. et al. (1994) Nature 329:21583-21586.

55. Miller, J., Germain, R.N., Efficient cell surface expression of class II MHC molecules in the absence of associated invariant chain. J.Exp.Med. 164:1478-1489 (1986).

56. Sambrook, J., Fritsch, E. F., and Maniatis, T., In: Molecular Cloning: A Laboratory Manual, 2nd Edition (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), 1989.

57. Sanger, F., Nicklen, S. and Coulsen, A. R. Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977).

58. Southern, E. M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517 (1975).

59. Spurney, R.F.; Coffman, T.M. J. Pharmacol. Exp. Ther. 283(1):207-215 (Oct. 1997).

60. Weinshank, R. L.; Zgombick, J. M.; Macchi, M. J.; Branchek, T. A.; Hartig, P. R. Proc. Natl. Acad. Sci. U.S.A. 89(8):3630-3634 (1992).

61. Quick, M.W., Lester, H.A. Methods for expression of excitability proteins in *Xenopus* oocytes. Meth. Neurosci. 19:261-279 (1994).

62. Smith, K.E., Forray, C., Walker, M.W., Jones, K.A., Tamm, J.A., Bard, J., Branchek, T.A., Linemeyer, D.L., Gerald, C. Expression cloning of a rat hypothalamic galanin receptor coupled to phosphoinositide turnover. J. Biol. Chem. 272:24612-24616 (1997).

63. Salon, J.A. and Owicki, J.A. Real-time measurements of receptor activity: Application of microphysiometric techniques to receptor biology. Methods in Neuroscience 25. Receptor Molecular Biology Ed. S.C. Sealfon, Academic Press, pp. 201-224 (1996).

64. Berridge, M.J., Downes, C.P., Hanley, M.R., Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. Biochem. J. 206: 587-595 (1982).

65. Cullen, B., "Use of eukaryotic expression technology in the functional analysis of cloned genes", *Methods Enzymol.* **152**: 685-704 (1987).

66. Bush, et al., "Nerve growth factor potentiates bradykinin-induced calcium influx and release in PC12 cells", *J. Neurochem.* **57**: 562-574 (1991).

67. Gundersen, C.B., et al., "Serotonin receptors induced by exogenous messenger RNA in Xenopus oocytes" *Proc. R. Soc. Lond. B. Biol. Sci.* **219(1214)**: 103-109 (1983).

68. Lazareno, S. and Birdsall, N.J.M., "Pharmacological characterization of acetylcholine stimulated [35S]-GTPgS binding mediated by human muscarinic m1-m4 receptors: antagonist studies", *Br. J. Pharmacology* **109**: 1120-1127 (1993).

69. Takahashi, T., et al., "Rat brain serotonin receptors in Xenopus oocytes are coupled by intracellular calcium to endogenous channels." *Proc. Natl. Acad. Sci. USA* **84(14)**: 5063-5067 (1987).

70. Tian, W., et al., "Determinants of alpha-Adrenergic Receptor Activation of G protein: Evidence for a Precoupled Receptor/G protein State." *Molecular Pharmacology* **45**: 524-553 (1994).